

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
5 August 2004 (05.08.2004)

PCT

(10) International Publication Number
WO 2004/065417 A2

(51) International Patent Classification⁷: **C07K 16/00**,
16/22, 16/42

(21) International Application Number:
PCT/US2004/001844

(22) International Filing Date: 23 January 2004 (23.01.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/442,484 23 January 2003 (23.01.2003) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS FOR PRODUCING HUMANIZED ANTIBODIES AND IMPROVING YIELD OF ANTIBODIES OR ANTIGEN BINDING FRAGMENTS IN CELL CULTURE

(57) Abstract: The present invention provides methods for producing humanized antibodies and increasing the yield of antibodies and/or antigen binding fragments when produced in cell culture. In one aspect of the invention, at least one framework region amino acid residue of the variable domain is substituted by a corresponding amino acid from a variable domain consensus sequence subgroup that has the most sequence identity with the HVRI and/or HVR2 amino acid sequence of the variable domain. In another aspect, an amino acid is placed at a position proximal to a cys residue that participates in an intrachain variable domain disulfide bond that corresponds to an amino acid found at that position in a variable domain consensus sequence subgroup that has the most sequence identity with the HVR1 and/or HVR2 amino acid sequence of the variable domain.



WO 2004/065417 A2

**METHODS FOR PRODUCING HUMANIZED ANTIBODIES AND IMPROVING
YIELD OF ANTIBODIES OR
ANTIGEN BINDING FRAGMENTS IN CELL CULTURE**

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Field of the invention

The present invention generally concerns the production of antibodies or antigen binding fragments in cell culture. More specifically, the invention provides methods for improving the yield of recombinant antibodies or antigen binding fragments in cell culture.

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Background

Antibodies, particularly humanized antibodies, have become very useful for diagnostic and therapeutic purposes. Humanized antibodies are antibodies in which CDRs or hypervariable regions (HVRs) from a non-human antibody are combined with human framework regions to form an antigen binding molecule. This exchange is sometimes known as a "CDR swap". There are different ways of selecting human framework sequences for humanized antibodies. One method involves selecting a human variable domain sequence that has a very similar framework sequence to that of the non-human antibody that is the source of the CDRs. Another method involves using a human variable domain consensus sequence as the source of the human framework regions. Often, a straight CDR swap does not result in high affinity antigen binding molecules so that additional changes or modifications are required to improve binding affinity of the humanized antibody. The necessity of making additional modifications can make humanization of antibodies a very time consuming process. In addition, humanization may not result in an antibody that can be produced in high yield in cell culture.

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Some of the uses of antibodies require large quantities of full-length completely assembled antibodies. Many techniques are now available for producing antibodies recombinantly using a variety of host cell systems including *E. coli*, yeast, plant cells, insect cells, and mammalian cells. Eukaryotic and prokaryotic systems have been used in large-scale production of antibodies. In particular, *E. coli* provides a useful organism for the expression of antibodies, including engineered antibodies, such as humanized antibodies. There are several advantages to *E. coli* expression systems, including a well-studied and convenient gene technology which permits constructs to be made easily and directly expressed, and the relatively convenient and economical large-scale production of product made possible by the fast growth of *E. coli* and its comparatively simple fermentation.

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Full-length antibodies comprise two heavy chains linked together by disulfide bonds and two light chains, each light chain being linked to one of the heavy chains by a disulfide bond. Each chain has an N-terminal variable domain (V_H or V_L) and one or more constant domains at the C-terminus; the constant domain of the light chain is aligned with and

disulfide bonded to the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Each of the variable domains of the heavy and light chain includes framework regions (FRs) and hypervariable regions (HVRs) and an intrachain disulfide bond. (See e.g. Chothia et al., *J. Mol. Biol.* 186:651-663 (1985); Novotny and Haber, *Proc. Natl. Acad. Sci. USA* 82:4592-4596 (1985);
5 Padlar et al., *Mol. Immunol.*, 23(9): 951-960 (1986); and S. Miller, *J. Mol. Biol.*, 216:965-973 (1990). Antibody fragments are also often produced and include combinations of heavy and light chain variable domains so as to form an antigen binding site. Antibody fragments include, for example, Fab, Fab', F(ab')₂, Fv, scFv, Fd, and Fd' fragments.

10 Generally, antibody production in prokaryotes involves synthesis of the light and heavy chains in the cytoplasm followed by secretion into the periplasm where processing of the chains takes place. Alternatively, the heavy and light chains can be directed to accumulate in the cytoplasm where they typically form inclusion bodies. Folding of the light and heavy chains occurs in conjunction with assembly of the folded light and heavy
15 chains to form an antibody molecule. Multiple covalent and non-covalent interactions occur between and within the heavy and light chains during these folding and assembly processes. Antibody yield can be greatly affected by the efficiency and fidelity of these processes. Following synthesis of the heavy and light chains, protein aggregation or proteolysis can occur thereby reducing the yield of the antibody.

20 Production and stability of antibody fragments have been studied more extensively than that of full length antibodies. Often the stability and/or production yields of scFv or Fab fragments of natural antibodies produced in host cells have been found to be insufficient. Honneger et al., *J. Mol. Biol.*, 309:687-699 (2001). Stability of the antibody or antibody fragment when incubated under physiological conditions is important for therapeutic
25 efficacy *in vivo*. Production yields and folding efficiency are important to increase the yield of antibodies or antibody fragments for therapeutic use. The stability of scFv fragments is not always correlated with expression yield in the bacterial periplasm. Worn et al., *J. Mol. Biol.*, 305:989-1010 (2001). Some stable scFv fragments show only poor expression yields in bacterial periplasm and some mutations can affect *in vivo* folding efficiency but not
30 stability. Worn et al., *supra*. The many factors that affect the periplasmic expression yield and/or stability of scFv are not yet fully understood.

Some structural features thought to be involved in stability and/or *in vivo* folding of antibody fragments have been previously described. For example, the FR1 of antibody fragments has been found to influence *in vivo* folding of antibody fragments in bacteria. de
35 Haard et al., *Prot. Eng.*, 11:1267-1276 (1998). In particular, the data of de Haard et al. suggested that mutations at residue 6 in the heavy chain interfered with the correct folding of a scFv. de Haard et al. *supra*. Jung et al. have described four different conformations of

the FR1 based on the amino acids found at positions H6, H7 and H10 (H9). Jung et al. *J. Mol. Biol.*, 309:701 (2001). Mutations at these residues, especially at residue 6, that disrupt the FR1 conformation can have adverse effects on folding yields and stability of scFv. Jung et al. *supra*. Residue 6 in the heavy chain is also thought to contribute to the stability of Fab, Fv, and ScFv fragments lacking disulfide bonds. Langdyk et al., *J. Mol. Biol.*, 283:95 (1998). Disulfide bonds also contribute to the stability of antibody domains. When disulfide bonds are removed, the H6 residue helped to stabilize the scFv but not when the disulfide bond was restored. A number of other point mutations at residues have been described as stabilizing or destabilizing in specific scFv fragments. Worn et al., *supra*. However, the effect of a mutation at a specific residue in an antibody or antibody framework may be unpredictable and may or may not affect the in vivo folding efficiency. A mutation at a residue in one antibody or antibody fragment that is beneficial for folding efficiency and yield may not be beneficial in another.

Methods for producing high affinity humanized antibodies can be time consuming and result in an antibody that is not optimal for production in cell culture. Multiple factors affect the yield and/or stability of antibodies and/or antibody fragments when produced in cell culture. Many of these factors are not yet well understood and may be unpredictable. Thus, there remains a need for improving the process of producing humanized antibodies and for improving the yield of antibodies or antibody fragments in cell culture, especially bacterial cell culture.

SUMMARY OF THE INVENTION

The present invention concerns methods for improving the process of humanizing an antibody or antigen binding fragment and for improving the yield of antibodies or antigen binding fragments in cell culture, especially bacterial cell culture. The invention is based on the discovery that the primary sequence of antibody variable domains can be designed or modified to contribute to correct folding, assembly, and yield of antibodies or antigen binding fragments. The invention involves identifying not only which residues should be substituted but also identifying which substitutions to make at those residues in a more predictable manner to result in improved yield of the antibodies.

One method for humanizing antibodies involves combining HVRs from a non-human antibody with human consensus framework regions that were derived from the most commonly occurring heavy and light chain subgroups in the sequence compilation of Kabat et al, Sequences of Proteins of Immunological Interest, NIH 1991. It has been discovered that selecting the most commonly occurring heavy and light chain consensus sequences may not provide an antibody that can be produced in high yield in cell culture. In one embodiment, the invention provides a method for selecting a human subgroup consensus

sequence for at least one of the framework region or regions based on identifying the subgroup consensus sequence that has the most sequence identity with the HVR1 and/or HVR2 sequence of the non-human antibody. The method may decrease the time to prepare a humanized antibody or antigen binding fragment that can be produced in high yield in cell culture.

In some embodiments, the method of producing a recombinant or humanized antibody or antigen binding fragment comprises expressing a variable domain comprising at least one FR sequence from a selected human subgroup variable domain consensus sequence, and a HVR1 and/or HVR2 sequence of a non-human antibody in a host cell, wherein the selected human subgroup consensus sequence is the human subgroup consensus sequence that has a HVR1 and/or HVR2 sequence that has the most sequence identity to the HVR1 and/or HVR2 of the non-human antibody; and recovering the antibody variable domain or an antibody or antigen binding fragment comprising the variable domain from the host cell.

In other embodiments, a method comprises comparing the HVR1 and/or HVR2 amino acid sequence of the non-human antibody with the corresponding HVR1 and/or HVR2 of the human consensus subgroups for the heavy or light chain, identifying the human variable domain subgroup consensus sequence with the most identity to that HVR1 and/or HVR2 amino acid sequence of the non-human antibody, and selecting at least one of the FRs of that subgroup consensus sequence as the FR sequence for the recombinant antibody or antigen binding fragment. A single selected FR or more than one FR selected from the group consisting of FR1, FR2, FR3, FR4 and mixtures thereof can be used to prepare the recombinant antibody. In one embodiment, a humanized antibody or antigen binding fragment includes the selected FR1 of the human heavy chain subgroup consensus sequence that has the most sequence identity with HVR1 and/or HVR2 of the non-human antibody. This method for improving the process of humanization may provide for a humanized antibody or antigen binding fragment that can be produced in high yield in cell culture in less time.

In one aspect of the invention, a method is provided for producing an antibody or antigen binding fragment in high yield in cell culture. A method comprises expressing a variable domain of the antibody or antigen binding fragment comprising at least one modified FR in a host cell, wherein the modified FR has a substitution of at least one amino acid position with a different amino acid, wherein the different amino acid is the amino acid found at the corresponding FR position of a human subgroup variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 sequence of the variable domain, wherein the antibody or antigen binding fragment variable domain comprising the modified

FR has improved yield in cell culture compared to an unmodified antibody or antigen binding fragment; and recovering the antibody or antigen binding fragment variable domain comprising the modified framework from the host cell.

5 In one aspect of the invention, a method is provided for preparing an antibody with improved yield when produced in cell culture. The method comprises expressing a variable domain of the antibody or antigen binding fragment comprising at least one modified FR in a host cell, wherein the modified FR is obtained by substituting at least one amino acid in a FR of a parent variable domain of the antibody or antigen binding fragment with a different amino acid; wherein the different amino acid is an amino acid found at the corresponding
10 FR position of a human subgroup variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity to a corresponding HVR1 and/or HVR2 amino acid sequence of the parent variable domain to form a modified FR. The antibody or antigen binding fragment having the modified FR has improved yield in a cell culture compared to an antibody or antigen binding fragment comprising the parent
15 variable domain and is recovered from the host cell.

In one embodiment, the method comprises comparing a) a HVR1 and/or HVR2 amino acid sequence of a variable domain of a parent antibody or antigen binding fragment to a corresponding HVR1 and/or HVR2 amino acid sequence of each of human subgroup variable domain consensus sequences and selecting the consensus sequence that has the
20 most sequence identity with the HVR1 and/or HVR2 sequence of the variable domain; b) identifying at least one amino acid in a FR of the variable domain that is different from an amino acid at a corresponding position of the selected human subgroup variable domain consensus sequence; and c) substituting the at least one amino acid identified in step (b) with the amino acid in the corresponding position of the selected subgroup variable domain
25 consensus sequence to form a variable domain with a modified FR.

At least one FR of a heavy or light chain variable domain or both can be modified or selected for use in accord with the methods of the invention. Modifications can be made in 1 FR or more than one FR selected from the group consisting of FR1, FR2, FR3, FR4 and mixtures thereof. Within a FR, at least one, and preferably more than one, amino acid
30 substitution is made in the FR. In one embodiment, all of the framework region residues of the parent antibody that are different than the selected subgroup consensus sequence are substituted with the amino acids found at those positions in the selected subgroup consensus sequence.

In another embodiment, the method comprises expressing a heavy chain variable
35 domain of the antibody or antigen binding fragment comprising at least one modified FR in a host cell, wherein the modified FR has a substitution of at least one amino acid position with a different amino acid, wherein the different amino acid is the amino acid found at the

corresponding FR position of a human heavy chain subgroup variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 sequence of the heavy chain variable domain, wherein the antibody or antigen binding fragment with the modified FR of the heavy chain has improved yield in cell culture compared to an unmodified parent antibody or antigen binding fragment; and recovering the antibody or antigen binding fragment variable domain comprising the modified framework from the host cell.

Another aspect of the invention provides another method for improving the yield of antibody or antigen binding fragment in culture. The method comprises modifying at least one FR sequence of a variable domain of the antibody or antigen binding fragment such that it is at least 50% identical in sequence to the corresponding FR sequence of a selected subgroup consensus sequence to form a modified FR, wherein the modified FR has a substitution of at least one amino acid position with a different amino acid, wherein the different amino acid is the amino acid found at the corresponding FR position of a selected human subgroup variable domain consensus sequence, wherein the selected human consensus subgroup sequence has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 sequence of the variable domain, wherein the antibody or antigen binding fragment with the modified FR has improved yield in cell culture compared to an unmodified parent antibody or antigen binding fragment; and recovering the variable domain with the modified FR.

Another aspect of the invention provides another method for producing an antibody or antigen binding fragment in high yield in cell culture. The method comprises expressing a modified variable domain of the antibody or antigen binding fragment in a host cell, wherein the modified variable domain has a substitution of at least one amino acid position proximal to a cys residue that participates in an intrachain variable domain disulfide bond with a different amino acid, wherein the different amino acid is the amino acid found at corresponding position of a human subgroup variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 amino acid sequence of the variable domain, wherein the antibody or antigen binding fragment comprising the modified variable domain has improved yield in cell culture compared to the antibody or antigen binding fragment; and recovering the antibody or antigen binding fragment comprising the modified variable domain from the host cell.

Another aspect of the invention provides another method for improving the yield of antibody or antigen binding fragment in culture. The method comprises: a) identifying at least one amino acid position in a first variable domain of a parent antibody or antigen binding fragment that is proximal to a cys residue that forms an intrachain variable domain

disulfide bond in the first variable domain; b) selecting a variable domain consensus sequence having the most sequence identity with a HVR1 and/or HVR2 amino acid sequence of the first variable domain; and c) placing at that position a different amino acid, wherein the different amino acid is an amino acid found at the corresponding position in the selected human subgroup consensus sequence to form a modified variable domain of the parent antibody or antigen binding fragment. In one embodiment, an antibody or antigen binding fragment whose sequence is known or readily ascertainable has at least one amino acid proximal to a cys residue substituted with the amino acid found at the corresponding position in the selected human subgroup consensus sequence. In one embodiment, at least one amino acid proximal to a cys residue can be modified and then incorporated into a humanized antibody or antigen binding fragment.

Another aspect of the invention involves expressing a polynucleotide molecule in a host cell. The method comprises expressing a polynucleotide encoding a variable domain of the antibody or antigen binding fragment comprising at least one modified FR in a host cell, wherein the modified FR has a substitution in at least one amino acid position in at least one FR with a different amino acid, wherein the different amino acid is the amino acid found at the corresponding FR position of a human subgroup variable domain consensus sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 amino acid sequence of the variable domain, wherein the antibody or antigen binding fragment comprising the modified FR region has improved yield in cell culture when compared to a parent unmodified antibody or antigen binding fragment; and recovering the antibody or antigen binding fragment comprising the modified FR from the host cell.

In one embodiment, the method comprises expressing a polynucleotide molecule encoding a modified variable domain of the antibody or antigen binding fragment in a host cell, wherein the modified variable domain has a substitution of at least one amino acid position proximal to a cys residue that participates in an intrachain variable domain disulfide bond with a different amino acid, wherein the different amino acid is the amino acid found at the corresponding position of a human subgroup variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 amino acid sequence of the variable domain, wherein the antibody or antigen binding fragment comprising the modified variable domain has improved yield as compared to an unmodified antibody or antigen binding fragment when produced in cell culture; and recovering the antibody or antigen binding fragment comprising the modified variable region from the host cell.

The invention also provides for antibody variable domains, antibodies, or antigen binding fragments that have modifications to the amino acid sequence to provide for increased yield when produced in cell culture.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the solubility differences between heavy and light chains produced in *E. coli*. Two different antibodies, anti-tissue factor antibody (A) and anti-VEGF antibody (B), were produced in *E. coli* and whole cell lysates were analyzed by SDS-PAGE under reducing conditions. Different fractions from the whole cell lysates were analyzed and compared to the total whole cell lysates. The location of heavy and light chains are shown. The arrows show that more heavy chains are found in the SDS/DTT soluble fraction and that light chains are mostly found in the soluble fraction. The first lane is the negative control; lane 1 is the whole cell-lysate; lane 2 is the soluble fraction; lane 3 is the SDS soluble fraction; and lane 4 is the SDS/DTT soluble fraction.

Figure 2 schematically illustrates the construction of a separate cistron vector for expression of a full-length antibody.

Figure 3 shows that substitution of heavy chain (HC) FR1 subgroup III amino acid residues with FR1 subgroup I amino acid residues in anti-VEGF VNERK antibody improves assembled antibody yield in *E. coli*. The yield of heavy and light chains is shown in panel A and the yield of assembled antibody products is shown in panel B. Whole cell lysates were prepared under reducing (A) and non-reducing (B) conditions and were analyzed by SDS PAGE immunoblot. Lane 1 is anti-VEGF VNERK with HC FR1 subgroup III sequence (HCFR1 = SGIII); lane 2 is anti-VEGF VNERK with HC FR1 subgroup I sequence (HCFR1 = SGI); lane 3 is anti-VEGF VNERK with HC FR1 subgroup II sequence (HCFR1 = SGII); and lane 4 is the negative control. In Figure 3A, the samples were prepared under reducing conditions, and positions of the heavy and light chains are identified. In Figure 3B, the samples were prepared under non-reducing conditions and the figures to the right of the gel show positions of completely assembled (at the top) and partially assembled antibody products.

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Figure 4 shows that substitution of HC FR1 subgroup III amino acid residues with subgroup I amino acid residues in anti-VEGF Y0317 antibody improves assembled antibody yield in *E. coli*. The yield of heavy and light chains is shown in panel A and the yield of assembled antibody products is shown in panel B. Whole cell lysates were prepared under reducing (A) and non-reducing (B) conditions and were analyzed by SDS PAGE immunoblot. Lane 1 is Y0317 antibody with HC FR1 subgroup III (HCFR1 = SGIII); and Lane 2 is Y0317 antibody with HC FR1 subgroup I (HCFR1 = SGI). In panel A, positions

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of the heavy and light chains are identified. In panel B, the figures to the right of the gel show position of completely assembled (at the top) and partially assembled antibody products.

5 Figure 5 shows that substitution of HC FR1 subgroup III amino acid residues with FR1 subgroup II amino acid residues in anti-IgE E25 antibody improves assembled antibody yield. The yield of heavy and light chains is shown in panel A and the yield of assembled antibody products is shown in panel B. Whole cell lysates were prepared under reducing (A) and non-reducing (B) conditions and were analyzed by SDS PAGE immunoblot. Lane 10 1 is negative control; Lane 2 is "wild type" antibody with HC FR1 subgroup III sequence (HCFR1 = SGIII*); and Lane 3 is antibody with HC FR1 subgroup III sequence replaced with the subgroup II sequence (HCFR1 = SGII). In panel A, positions of heavy and light chains are indicated. In panel B, the figures to the right of the gel show completely assembled (at the top) and partially assembled antibody products. The asterisk indicates that 15 wild type antibody E25 includes a change at position 24 from an alanine to a valine at that position. Valine at position 24 corresponds to the amino acid at that position in the murine sequence and in the human consensus subgroup II sequence.

 Figure 6 shows the assembled antibody yields of anti-VEGF antibodies with single 20 amino acid substitutions in HC FR1 replacing subgroup III residues with the subgroup I residue at each position. The yield of heavy and light chains is shown in panels A and B and the yield of assembled antibody products is shown in panels C and D. Whole cell lysates were prepared under reducing (A, B) and non-reducing (C, D) conditions and were analyzed by SDS PAGE immunoblot. In panels A and C, lane 1 is the wt anti-VEGF with 25 HC FR1 subgroup III sequence; lane 2 is an antibody with a substitution E1Q; lane 3 is an antibody with an E6Q substitution; lane 4 is an antibody with a G9A substitution; lane 5 is an antibody with G10E substitution; lane 6 is an antibody with a L11V substitution; lane 7 is an antibody with V12K substitution; and lane 8 is an antibody with the HC FR1 subgroup I; lane 9 is the negative control. In panels B and D, lane 1 is the wild type with HC FR1 30 subgroup III; lane 2 is an antibody with Q13K; lane 3 is an antibody with G16A substitution; lane 4 is an antibody with L18V substitution; lane 5 is an antibody with R19K substitution; lane 6 is an antibody with L20V substitution; lane 7 is an antibody with A23K substitution; lane 8 is an antibody with HC FR1 subgroup I; and lane 9 is a negative control.

35 Figure 7 shows the assembled antibody yields of anti-VEGF antibodies with single amino acid substitutions in the heavy chain FR1 replacing subgroup III residues with the subgroup I residue at those positions. The yield of heavy and light chains is shown in panel

A and the yield of assembled antibody products is shown in panel B. Whole cell lysates were prepared under reducing (A) and non-reducing (B) conditions and were analyzed by SDS PAGE immunoblot. Lane 1 is negative control; lane 2 is the anti-VEGF VNERK wt with HC FR1 subgroup III sequence (HCFR1 = SGIII); lane 3 is the anti-VEGF antibody with HC FR1 subgroup I sequence (HCFR1 = SGI); lane 4 is an antibody with E1Q substitution; lane 5 is an antibody with E6Q substitution; lane 6 is an antibody with L11V substitution; and lane 7 is an antibody with A23K substitution.

Figure 8 shows the yield of assembled antibody products (B) and the heavy and light chains (A) of anti-VEGF first generation wild-type antibodies. The antibodies have single or double amino acid substitutions at positions proximal to cys residues that form an intrachain disulfide bond. Whole cell lysates were prepared under reducing (A) and non-reducing (B) conditions and were analyzed by SDS PAGE immunoblot. Lane 1 is a negative control; lane 2 is the first generation wild type anti-VEGF antibody; lane 3 is an antibody with a M4L substitution in the light chain; lane 4 is an antibody with a F71Y substitution in the light chain; lane 5 is an antibody with a M34I substitution in the heavy chain; and lane 6 is an antibody with E6Q and M34I substitutions in the heavy chain.

Figure 9 shows the yield of heavy and light chains (A) and assembled antibody products (B) for anti-VEGF antibodies with a modified variable domain. The antibodies have double or single amino acid substitutions in positions proximal to cys residues that form an intrachain disulfide bond. Whole cell lysates were prepared under reducing (A) and non-reducing (B) conditions and were analyzed by SDS PAGE immunoblot. Lane 1 is negative control; lane 2 is anti-VEGF first generation wt; lane 3 is an antibody with a substitution M4L in the light chain and a substitution M34I in the heavy chain; lane 4 is an antibody with a M4L substitution in the light chain; and lane 5 is an antibody with a M34I substitution in the heavy chain.

Figure 10 shows the yield of heavy and light chains (A) and assembled antibody products (B) of anti-VEGF VNERK antibodies with a modified variable domain. The antibodies have single amino acid substitutions at position 78 in the heavy chain replacing the subgroup III (L78) residue with either the subgroup I (A78) or the subgroup II residue (F78). Whole cell lysates were prepared under reducing (A) and non-reducing (B) conditions and were analyzed by SDS PAGE immunoblot. Lane 1 is a negative control; lane 2 is anti-VEGF antibody with a HC subgroup I residue at position 78 (A78-SGI); lane 3 is anti-VEGF antibody with a HC subgroup III residue at position 78, (L78-SGIII); and lane 4 is an antibody with a HC subgroup II residue at position 78 (F78-SGII).

Figure 11 shows the yield of heavy and light chains (A) and assembled antibody products (B) of anti-VEGF antibody YO317 with a single amino acid substitution at position 34 in the heavy chain. Whole cell lysates were prepared under reducing (A) and non-reducing (B) conditions and were analyzed by SDS PAGE immunoblot. Lane 1 is a negative control; lane 2 is anti-VEGF YO317 with HC FR1 subgroup III; and lane 3 is YO317 antibody with M34I substitution in heavy chain.

Figure 12 shows the yield of heavy and light chains (A) and assembled antibody products (B) of anti-VEGF antibody YO317 antibody with an amino acid substitution F71Y in the light chain. Whole cell lysates were prepared under reducing (A) and non-reducing (B) conditions and were analyzed by SDS PAGE immunoblot. Lane 1 is a negative control; lane 2 is anti-VEGF antibody YO317; lane 3 is an antibody with F71Y substitution in the light chain.

Figure 13 shows the yield of assembled antibody products of anti-VEGF VNERK antibodies with modified framework regions. The samples were prepared under non-reducing conditions and run on a SDS PAGE gel. The gels were stained with Coomassie Blue (A) or detectably labeled anti-Fc antibody (B). Lane 1 is a negative control; Lane 2 is the anti-VEGF antibody VNERK with HC subgroup III framework residues, except for those changed as a part of humanization (HCFR = SGIII); lane 3 is anti-VEGF VNERK with HC FR1 residues from heavy chain subgroup I consensus sequence; lane 4 is anti-VEGF VNERK with HC FR1 and FR2 region residues from heavy chain subgroup I; and lane 5 is anti-VEGF VNERK antibody with HC FR1, FR2 and FR3 residues of heavy chain consensus sequence subgroup I.

Figure 14 shows the yield of assembled antibody products of anti-VEGF VNERK antibodies prepared by large scale fermentation. The samples were prepared under non-reducing conditions and run on a SDS PAGE gel. The gels were stained with Coomassie Blue (A) or detectably labeled anti-Fab antibody (B). The anti-VEGF antibody with HC FR1 sequences from consensus subgroup I was prepared by large-scale fermentation on two separate occasions (one batch was labeled AB249; the other batch was labeled AB444). Lane 1 is anti-VEGF VNERK antibody with HC FR1 subgroup III (AB422); lane 2 is anti-VEGF VNERK with HC FR1 subgroup I batch AB249; and lane 3 is anti-VEGF VNERK with HC FR1 subgroup I batch AB444.

Figure 15 A-D shows the polynucleotide sequence (SEQ ID NO: 4) encoding an amino acid sequence (SEQ ID NO: 5) of heavy and light chains of anti-VEGF antibody VNERK in plasmid pxVG11VNERK. The positions of cys residues that form an intravari-
5 domains.

Figure 16A-D shows the polynucleotide sequence (SEQ ID NO: 6) encoding an amino acid sequence (SEQ ID NO: 7) of the heavy and light chains of anti-VEGF antibody YO317 in plasmid pxVG2AP11.

Figure 17 A-D shows the polynucleotide sequence (SEQ ID NO: 8) encoding an amino acid sequence of (SEQ ID NO: 9) of heavy and light chains of anti-VEGF VNERK with heavy chain FR1 subgroup I consensus sequence in plasmid pVKFR1-2. The positions of cys residues that form an intravari-
15 domains.

Figure 18 A-D shows the polynucleotide sequence (SEQ ID NO: 10) encoding an amino acid sequence (SEQ ID NO: 11) of the heavy and light chains of anti-VEGF antibody VNERK with heavy chain FR1 subgroup II consensus in plasmid pVKSGII. The position of cys residues that form an intravari-
20 domains.

Figure 19 A-D shows the polynucleotide sequence (SEQ ID NO: 12) encoding an amino acid sequence (SEQ ID NO: 13) of the heavy and light chains of anti-VEGF antibody YO317 with heavy chain FR1 subgroup I consensus sequence in plasmid pYOFR1-2.

Figure 20 A-D shows the polynucleotide sequence (SEQ ID NO: 20) encoding an amino acid sequence (SEQ ID NO: 21) of heavy and light chains of anti-IgE antibody E25 in pE25-11.

Figure 21 A-D shows the polynucleotide sequence (SEQ ID NO: 22) encoding an amino acid sequence (SEQ ID NO: 23) of heavy and light chains of anti-IgE antibody E25 with heavy chain FR1 subgroup II consensus sequence in plasmid pE25-SGII.

Figure 22 A-C shows the polynucleotide sequence (SEQ ID NO: 24) encoding an amino acid sequence (SEQ ID NO: 25) of heavy and light chains of anti-VEGF first
35 generation wild type in plasmid pVG50.

Figure 23 A-E shows the polynucleotide sequence (SEQ ID NO: 26) encoding an amino acid sequence (SEQ ID NO: 27) of the heavy and light chains of anti-VEGF VNERK antibodies in plasmid pVKSGI with FR 1, 2, 3, and 4 with a subgroup I consensus sequence.

5

Table of Sequences

SEQ ID NO:	Name	Sequence	Page
1	FR1 Subgroup I	QVQLVQSGAEVKKPGASVKVSKAS	63, 64, 65
2	FR1 Subgroup II	QVQLQESGPGLVKPSQTLSTCTVS	63
3	FR1 Subgroup III	EVQLVESGGGLVQPGGSLRLSCAAS	63
4	Heavy and light chains of VNERK in pxVG11VNERK	polynucleotide sequence	Fig. 15
5	Heavy and light chains of VNERK in pxVG11VNERK	amino acid sequence	Fig. 15
6	Heavy and light chains of YO317 in pxVG2AP11	polynucleotide sequence	Fig. 16
7	Heavy and light chains of YO317 in pxVG2AP11	amino acid sequence	Fig. 16
8	Heavy and light chains of VNERK with heavy chain FRI SGI in pVKFR1-2	polynucleotide sequence	Fig. 17
9	Heavy and light chains of VNERK with heavy chain FRI SGII in pVKFR1-2	amino acid sequence	Fig. 17
10	Heavy and light chains of VNERK with heavy chain FRI SGII in pVKSGII	polynucleotide sequence	Fig. 18
11	Heavy and light chains of VNERK with heavy chain FRI SGII in pVKSGII	amino acid sequence	Fig. 18
12	Heavy and light chains of YO317 with heavy chain FRI SGI in pYOFR1-2	polynucleotide sequence	Fig. 19
13	Heavy and light chains of YO317 with heavy chain FRI SGI in pYOFR1-2	amino acid sequence	Fig. 19
14	VNERK heavy chain HVR1 residues 26-35	GYTFTNYGIN	33, 72, 73
15	Human heavy chain consensus sequence	GYTFTSYAIS	33, 72, 74

SEQ ID NO:	Name	Sequence	Page
	from Subgroup I		
16	Human heavy chain consensus sequence from Subgroup II	GGSVSSYWSWN	33, 72, 74, 76
17	Human heavy chain consensus sequence from Subgroup III	GFTFSSYAMS	33, 72, 74, 76
18	YO317 heavy chain HVR1 residues 26-35	GYDFTHYGMN	74
19	E25 heavy chain HVR1 residues 26-35	GYSITSGYSWN	76
20	Heavy and light chains of anti-IgE antibody E25 in pE25-11	polynucleotide sequence	Fig. 20
21	Heavy and light chains of anti-IgE antibody E25 in pE25-11	amino acid sequence	Fig. 20
22	Heavy and light chains of anti-IgE antibody E25 with heavy chain FR1 SGII in pE25-SGII	polynucleotide sequence	Fig. 21
23	Heavy and light chains of anti-IgE antibody E25 with heavy chain FR1 SGII in pE25-SGII	amino acid sequence	Fig. 21
24	Heavy and light chains of anti-VEGF first generation wild type in pVG50	polynucleotide sequence	Fig. 22
25	Heavy and light chains of anti-VEGF first generation wild type in pVG50	amino acid sequence	Fig. 22
26	Heavy and light chains of VNERK in pVKSGI with FR1, 2, 3, and 4 SGI consensus sequence	polynucleotide sequence	Fig. 23
27	Heavy and light chains of VNERK in pVKSGI with FR1, 2, 3, and 4 SGI consensus sequence	amino acid sequence	Fig. 23
28	heavy chain FR2 Subgroup III consensus sequence	WVRQAPGKGLEWVS	73
29	heavy chain FR2 anti-VEGF VNERK sequence	WVRQAPGKGLEWVG	73
30	heavy chain FR3	RFTISRDNSKNTLYLQMNSLRAEDTAVY	73

SEQ ID NO:	Name	Sequence	Page
	Subgroup III consensus sequence	YCAR	
31	heavy chain FR3 anti-VEGF VNERK sequence	RFTFSLDTSKSTAYLQMNSLRAEDTAVY YCAK	73
32	heavy chain FR4 Subgroup III consensus sequence	WGQGTLVTVSS	73

DETAILED DESCRIPTION

5 The numbering system of all of the antibodies described herein is according to Kabat et al. (1991) *Sequence of Proteins of Immunological Interest*, 4th Ed. National Institute of Health, Bethesda, MD.

A. Definitions

10 The terms "antibody" and "immunoglobulin" are used interchangeably in the broadest sense and include monoclonal antibodies (full-length or intact monoclonal antibodies), polyclonal antibodies, humanized, multivalent antibodies, and multispecific antibodies (*e.g.*, bispecific antibodies so long as they exhibit the desired biological activity). A naturally occurring antibody comprises four polypeptide chains, two identical heavy (H) chains and two identical light (L) chains inter-connected by disulfide bonds. Each heavy

15 chain is comprised of a heavy chain variable region domain (V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region domain (V_L) and a light chain constant region domain. The light chain constant region is comprised of one domain, C_L . The V_H and V_L domains can be further subdivided into hypervariability regions (HVR),

20 interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three HVRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, HVR1, FR2, HVR2, FR3, HVR3, FR4.

 Depending on the amino acid sequences of the constant domains of their heavy chains, antibodies (immunoglobulins) can be assigned to different classes. There are five

25 major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG-1, IgG-2, IgA-1, IgA-2, and etc. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α , δ , ϵ , γ , and μ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described

30 generally in, for example, Abbas et al., *Cellular and Mol. Immunology*, 4th ed. (2000). An

antibody may be part of a larger fusion molecule, formed by covalent or non-covalent association of the antibody with one or more other proteins or peptides.

5 The light chains of antibodies from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

The terms "full-length antibody," "intact antibody" and "whole antibody" are used herein interchangeably, to refer to an antibody in its substantially intact form including at least 2 heavy and 2 light chains, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains that contain Fc region. A full-length
10 antibody can be a native sequence antibody or a recombinant antibody. A full-length antibody can be human, humanized and/or affinity matured.

The term "parent antibody", "parent antigen binding fragment" or "unmodified variable domain" are used interchangeably and as used herein refer to an antibody or antigen binding fragment that provides variable domain sequences that are the source material for a
15 method of producing a humanized antibody or for modification in accord with the methods of the invention. Parent antibody or antigen binding fragment variable domain sequences are known or can readily be ascertained using methods known in the art. A parent antibody includes but is not limited to a humanized antibody, human antibody, monoclonal antibody, chimeric antibodies, polyclonal antibodies, multivalent antibodies and multispecific
20 antibodies. Human consensus subgroup sequences can also serve as a source of antibody variable domain sequences. Antigen binding fragments can include Fab fragments, Fab' fragments, Fd' fragment, Fv fragment, Fd fragment, F(ab')₂ fragment, dAb fragment, hingeless antibodies, single chain antibodies, diabodies, single arm antigen binding molecules comprising a light chain, a heavy chain and a N-terminally truncated heavy chain
25 constant region sufficient to form a Fc region capable of increasing the half life of the single arm antigen binding molecule, and linear antibodies.

The phrase "assembled antibody products or assembled antibody" as used herein refers to an antibody or antibody fragment that comprises at least one antibody variable domain, for example a light and a heavy chain variable domain, that forms an antigen
30 binding site. In some cases, the association may involve one or more interchain disulfide bonds. For example, a completely assembled full-length antibody refers to an antibody including 2 heavy and 2 light chains and is completely disulfide bonded so as to form the structure of a naturally occurring antibody.

"Antibody fragments" comprise only a portion of an intact antibody, generally
35 including an antigen binding site of the intact antibody and thus retaining the ability to bind antigen. Examples of antibody fragments encompassed by the present definition include: (i) the Fab fragment, having VL, CL, VH and CH1 domains having one interchain disulfide

bond between the heavy and light chain; (ii) the Fab' fragment, which is a Fab fragment having one or more cysteine residues at the C-terminus of the CH1 domain; (iii) the Fd fragment having VH and CH1 domains; (iv) the Fd' fragment having VH and CH1 domains and one or more cysteine residues at the C-terminus of the CH1 domain; (v) the Fv fragment having the VL and VH domains of a single arm of an antibody; (vi) the dAb fragment which consists of a VH domain; (vii) hingeless antibodies including at least VL, VH, CL, CH1 domains and lacking hinge region; (viii) F(ab')₂ fragments, a bivalent fragment including two Fab' fragments linked by a disulfide bridge at the hinge region; (ix) single chain antibody molecules (*e.g.* single chain Fv; scFv); (x) "diabodies" with two antigen binding sites, comprising a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain; (xi) single arm antigen binding molecules comprising a light chain, a heavy chain and a N-terminally truncated heavy chain constant region sufficient to form a Fc region capable of increasing the half life of the single arm antigen binding domain; (xii) "linear antibodies" comprising a pair of tandem Fd segments (VH-CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions.

A "biologically active" or "functional" immunoglobulin is one capable of exerting one or more of its natural activities in structural, regulatory, biochemical or biophysical events. For example, a biologically active antibody may have the ability to specifically bind an antigen and the binding may in turn elicit or alter a cellular or molecular event such as signaling transduction or enzymatic activity. A biologically active antibody may also block ligand activation of a receptor or act as an agonist antibody. The capability of a full-length antibody to exert one or more of its natural activities depends on several factors, including proper folding and assembly of the polypeptide chains. As used herein, the biologically active immunoglobulins generated by the disclosed methods are typically, but not necessarily, heterotetramers having two identical L chains and two identical H chains that are linked by multiple disulfide bonds and properly folded and assembled.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are essentially identical except for variants that may arise during production of the antibody.

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so

long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

“Humanized” forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region (HVR) of the recipient are replaced by residues from a hypervariable region (HVR) of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues to improve antigen binding affinity. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or the donor antibody. These modifications may be made to improve antibody affinity or functional activity. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. Humanized antibodies can also be produced as antigen binding fragments as described herein. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of or derived from a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also the following review articles and references cited therein: Vaswani and Hamilton, *Ann. Allergy, Asthma & Immunol.* 1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurle and Gross, *Curr. Op. Biotech* 5:428-433 (1994).

A “human antibody” is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen binding residues.

An “affinity matured” antibody is one with one or more alterations in one or more hypervariable regions which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al., *Bio/Technology* 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas et al., *Proc. Nat. Acad. Sci. USA* 91:3809-3813 (1994); Scier et al., *Gene* 169:147-

155 (1995); Yelton et al., *J. Immunol.* 155:1994-2004 (1995); Jackson et al., *J. Immunol.* 154(7):3310-9 (1995); and Hawkins et al., *J. Mol. Biol.* 226:889-896 (1992).

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions (HVR1, HVR2 and HVR3) both in the light chain (LC) and the heavy chain (HC) variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FRs (FR1, FR2, FR3 and FR4, respectively), largely adopting a β -sheet configuration, connected by three hypervariable regions. The β -sheet structure formed by the FRs are also connected to one another by an intravari-
 10 domain disulfide bond. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen binding site of antibodies (see Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991), pages 647-669). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity, and long half-life
 15 through size and FcRn binding.

The term "hypervariable region" (hereinafter "HVR") when used herein refers to the amino acid residues of an antibody variable domain the presence of which are necessary for antigen binding. Each variable domain typically has three HVR regions identified as HVR1, HVR2 and HVR3. Each hypervariable region comprises amino acid residues from a "complementarity determining region" (hereinafter "CDR") (*i.e.* about residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (*i.e.* about
 25 residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). In some instances, a hypervariable region can include amino acids from both a CDR region and a hypervariable loop. For example, the hypervariable region I (HVR1) of the heavy chain can include amino acids 26 to 35.

35 "Framework regions" (hereinafter FR) are those variable domain residues other than the hypervariable region residues. Each variable domain typically has four FRs identified as FR1, FR2, FR3 and FR4. If the hypervariable regions comprise amino acid

residues from CDRs, the light chain FR residues are positioned at about residues 1-23 (LCFR1), 35-49 (LCFR2), 57-88 (LCFR3), and 98-107 (LCFR4) and the heavy chain FR residues are positioned about at residues 1-30 (HCFR1), 36-49 (HCFR2), 66-94 (HCFR3), and 103-113 (HCFR4) in the heavy chain residues. If the hypervariable regions comprise amino acid residues from hypervariable loops, the light chain FR residues are positioned about at residues 1-25 (LCFR1), 33-49 (LCFR2), 53-90 (LCFR3), and 97-107 (LCFR4) in the light chain and the heavy chain FR residues are positioned about at residues 1-25 (HCFR1), 33-52 (HCFR2), 56-95 (HCFR3), and 102-113 (HCFR4) in the heavy chain residues. In some instances, when the hypervariable region comprises amino acids from both a CDR and hypervariable loop, the FR residues will be adjusted accordingly. For example, when HC HVR1 includes amino acids H26-H35, the heavy chain FR1 residues are at positions 1-25 and the FR2 residues are at positions 36-49.

The term "human variable domain subgroup consensus sequence" refers to an artificial amino acid sequence for a variable region that is generally not obtained from any single naturally occurring immunoglobulin. Sequences of naturally occurring immunoglobulins have been compiled and analyzed, for example, by Kabat et al. supra or at immuno-bme-nwu-edu. The variable domain sequences have been placed in subgroups based on similarity of the sequences. For example, human heavy chain variable domain sequences identified by Kabat can be categorized into three subgroups identified as subgroup I, subgroup II and subgroup III. The consensus sequence is an artificial sequence that is derived from a comparison of the amino acid sequences of known human immunoglobulin variable region sequences in a subgroup. Using this comparison, an amino acid variable region sequence is derived to form a consensus, or average, of the sequences of the natural immunoglobulins in the subgroup. A consensus amino acid sequence is a sequence that at each position has an amino acid found most frequently in known immunoglobulins in the subgroup. Useful consensus sequences include variable domain consensus sequences derived from the data provided in Kabat et al., 1991, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD and variants thereof.

The term "corresponding" or "corresponds" refers to an amino acid position or amino acid sequence that is found at the same position or positions in a sequence when the sequence is aligned with a reference sequence. Typically, the amino acid sequences are aligned using the same numbering system in each sequence.

A sequence has the "most sequence identity" if it has an amino acid sequence that has the greatest degree of sequence identity to a reference sequence when the two sequences are aligned. The percent identity between two sequences can be determined by aligning the two sequences, accounting for gaps as necessary to achieve the best alignment and

determining the number of residues that are the same in both sequences divided by the number of amino acids in the reference sequence. A number of computer programs can also be utilized for the purposes of aligning sequences and determining the % identity. For example, a heavy chain HVR1 amino acid sequence from an antibody or antigen binding
5 fragment is compared to the corresponding HVR1 sequence of a heavy chain subgroup consensus sequence and the number of amino acids that are the same is divided by the number of amino acids in the HVR1 subgroup consensus sequence resulting in the % identity. The comparison is repeated with other heavy chain consensus sequences and the heavy chain subgroup consensus sequence which has the most % sequence identity is
10 selected.

The phrase "proximal to a cys residue that forms an intrachain disulfide bond" refers to an amino acid position in a three-dimensional structure that is located near a cys residue that forms an intrachain variable domain disulfide bond or an amino acid position adjacent to a cys residue that forms an intrachain disulfide bond. Each variable domain of
15 an antibody or antigen binding fragment typically has a single intrachain disulfide bond formed between 2 cys residues. In many antibodies the position of these cys residues is conserved. The cys residues are usually found at positions L23 and L88 in the light chain and at position H22 and H92 in the heavy chain.

Amino acid positions adjacent to the cys residues are the two amino acid positions
20 on either side of the cys residue in a linear sequence. For example, in the heavy chain variable domain where the cys residue is at position 22, adjacent residues are at positions 20 and 21 and positions 23 and 24 in the linear sequence.

An amino acid position in a three dimensional structure that is near to a cys residue is a position where a side chain (or in the case of Gly, an alpha carbon) of the amino acid in
25 that position is about 5 angstroms or less from the cys residue or is a position where the amino acid in that position has lost about 10 square angstroms or more of solvent accessible surface area by contacting the cys residue. Amino acid positions proximal to a cys residues can be determined by analysis of the crystal structure of the antibody or a three dimensional molecular model of the antibody using computer programs such as MIDAS (UCSF) and
30 solvent accessible surface areas can be calculated using programs such as SOLV (G. S. Smith (1985) "A computer program for the calculation of the molecular volume and surface area of proteins", Merck, Sharpe and Dohme Res. Laboratories, QCPE).

The term "improved yield" refers to an increase in the amount of assembled antibody product with modified framework regions or variable domains when compared to
35 an unmodified or control antibody product that is prepared under similar conditions in similar cell types. The yield of antibodies or antigen binding fragment modified in accord with the methods of the invention is increased at least about 2 fold or more, more preferably

about 2 to 4 fold, more preferably about 2 to 8 fold, and most preferably 2 to 16 fold when compared to the yield of an unmodified or control antibody. Relative yields of antibody products can be determined using standard methods including scanning densitometry of SDS-PAGE gels and/or immunoblots and the AME5-RP assay.

5 The term “improved folding efficiency” refers to an improved ability of an antibody or antigen binding fragment to completely fold and assemble when produced in a cell culture. For example, a full-length IgG antibody is completely assembled and folded when it forms a molecule comprised of 2 heavy and 2 light chains connected by 4 interchain disulfide bonds. An antibody has improved folding efficiency when the amount of
10 completely assembled antibody product is increased while the expression level of the heavy and light chains remains about the same compared to a reference or control antibody that has not been modified as determined by methods such as SDS-PAGE, immunoblot analysis, and scanning densitometry as described herein.

 “Affinity binding” refers to the strength of the sum total of noncovalent interactions
15 between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen or FcRn receptor). The affinity of a molecule X for its partner Y is represented by the dissociation constant (K_d), which can generally be determined by using methods known in the art.

 The term “vector,” as used herein, is intended to refer to a nucleic acid molecule
20 capable of transporting another nucleic acid to which it has been linked. One type of vector is a “plasmid”, which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a phage vector. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which
25 they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are
30 referred to herein as “recombinant expression vectors” (or simply, “recombinant vectors”). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, “plasmid” and “vector” may be used interchangeably as the plasmid is the most commonly used form of vector.

 The term “cistron,” as used herein, is intended to refer to a genetic element broadly
35 equivalent to a translational unit comprising the nucleotide sequence coding for a polypeptide chain and adjacent control regions. “Adjacent control regions” include, for example, a translational initiation region and a termination region.

A "separate cistron" expression vector according to the present invention refers to a single vector comprising at least two separate promoter-cistron pairs, wherein each cistron is under the control of its own promoter. Upon expression of a separate cistron expression vector, both transcription and translation processes of different genes are separate and
5 independent.

The term "host cell" (or "recombinant host cell"), as used herein, is intended to refer to a cell that has been genetically altered, or is capable of being genetically altered by introduction of an exogenous polynucleotide molecule, such as a recombinant plasmid or vector. It should be understood that such terms are intended to refer not only to the
10 particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein.

A "disorder" is any condition that would benefit from treatment with the antibody.
15 This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include malignant and benign tumors, non-leukemias and lymphoid malignancies; neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory,
20 angiogenic and immunologic disorders.

As used herein, "treatment" refers to clinical intervention in an attempt to alter the natural course of the individual or cell being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include preventing occurrence or recurrence of disease, alleviation of symptoms,
25 diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis.

Modes for Carrying out the Invention

30 The present invention concerns methods for improving the process of humanization and improving the yield of recombinantly produced antibodies or antigen binding fragments in cell culture, especially bacterial cell culture. Bacterial systems have been widely used for expressing antibody fragments, but there have been few attempts to express and recover functional completely assembled full-length antibodies in high yield. Because of the
35 complex structure and large size of completely assembled full-length antibodies, it is often difficult to achieve proper folding and assembly of the expressed heavy and light chains, especially in bacterial cells. The invention is based on the discovery that the sequence of

antibody variable domains can be designed or modified to contribute to correct folding and assembly of antibodies or antibody fragments, and thereby increase yield. The invention involves identifying not only which residues should be substituted but also identifying which substitutions to make at those residues in a more predictable manner to result in improved yield of the antibodies or antigen binding fragments.

In particular, it has now been discovered that folding, assembly, and yield of antibodies or antigen binding fragments is improved by substituting at least one amino acid residue of a framework region of a variable domain with an amino acid residue found at the corresponding position in the framework region of the human variable domain subgroup consensus sequence with the most sequence identity with the HVR1 and/or HVR2 amino acid sequence of the variable domain. In one embodiment of the invention, the FR1 sequence of the heavy chain variable domain is substituted with FR1 amino acid sequence of the human heavy chain variable domain subgroup consensus sequence with the most sequence identity to the HVR1 sequence of the antibody. In another aspect, at least one amino acid position proximal to a cys residue that forms an intrachain disulfide bond is substituted with an amino acid at the corresponding position in the human variable domain subgroup consensus sequence with the most identity to the HVR1 and/or HVR2 amino acid sequence of the antibody. While the processes of the invention for improving yield of antibodies in cell culture have been illustrated for production of full-length completely assembled antibodies, it should be understood that the approach described herein is applicable to the production of antigen binding fragments.

This approach is also useful for designing and producing recombinantly produced antibodies, such as humanized antibodies or antigen binding fragments, to include features that enhance yield of antibody when produced in cell culture. For example, when the desired HVR1 and/or HVR2 amino acid sequence of the recombinant antibody is identified, the human variable domain subgroup consensus sequence with the most identity to that HVR1 and/or HVR2 amino acid sequence can be selected and at least one of the FRs of that subgroup selected for the FR sequence of the recombinant antibody or antigen binding fragment. In another aspect, the variable domain of the humanized antibody or antigen binding fragment can be formed or modified so that at least one amino acid position proximal to a cys residue that forms an intrachain disulfide bond has a substitution with an amino acid at the corresponding position in the human variable domain subgroup consensus sequence with the most identity to the HVR1 and/or HVR2 amino acid sequence of the antibody. The methods for improving the process of humanization may provide for a humanized antibody or antigen binding fragment that can be produced in high yield in cell culture in less time.

Parent Antibody Variable Domains

The present invention is applicable to modification of antibodies including monoclonal antibodies (full-length or intact monoclonal antibodies), polyclonal antibodies, humanized, multivalent antibodies, and multispecific antibodies (*e.g.*, bispecific antibodies so long as they exhibit the desired biological activity). The methods of the invention can also be employed for improving the yield of antigen binding fragments of antibodies including Fab, Fab', Fd, Fd', Fv, dAb, F(ab')₂, scFv, scFv₂, single chain antibodies, hingeless antibodies, diabodies, a single arm antigen binding molecule comprising one light chain, one heavy chain and a -terminally truncated heavy chain constant region sufficient to form a Fc region capable of increasing the half life of the single arm antigen binding molecule, and linear antibodies.

The source or parent antibody variable domains useful in the methods of the invention include those from naturally occurring and/ or synthetic antibodies including monoclonal antibodies, humanized antibodies, chimeric antibodies, antibody variable domains produced by phage display, and human antibodies. Human antibodies may be obtained from transgenic animals having the human immune system such as produced by Abgenix (See, *e.g.*, U.S. Pat. Nos. 5,591,669, 5,589,369, 5,545,807, and 6,075,181).

In one embodiment, the source or parent antibody variable domains for the HVRs is a non-human monoclonal antibody and the source or parent antibody variable domain for the FR sequences is a human antibody or human subgroup consensus sequences. The FR sequences are preferably consensus framework region sequences from a human subgroup of antibody variable domain sequences. The HVR1 and/ or HVR2 of the non-human monoclonal antibody are compared to the corresponding HVR1 and/or HVR2 sequences of the human subgroup consensus sequences, and the human consensus subgroup with the most sequence identity is selected to provide at least one of the framework region sequences in the humanized antibody or antigen binding fragment.

The antibodies or antigen binding fragments modified in accord with the methods of the invention have antigen binding specificity. Preferably, the antibodies of the invention are specific to antigens that are biologically important polypeptides. More preferably, the antibodies of the invention are useful for therapy or diagnosis of diseases or disorders in a mammal. Full-length antibodies made according to the present invention are particularly useful as therapeutic antibodies such as blocking antibodies, agonist antibodies or antibody conjugates. Non-limiting examples of therapeutic antibodies include anti-VEGF, anti-IgE, anti-CD11, anti-CD18, anti-CD40, anti-tissue factor (TF), anti-HER2, and anti-TrkC antibodies. Antibodies directed against non-polypeptide antigens (such as tumor-associated glycolipid antigens) are also contemplated.

Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or a ligand such as a growth factor. Exemplary antigens include molecules such as renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIc, factor IX, tissue factor (TP), and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1 -alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF- β ; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF- α and TGF- β , including TGF- β 1, TGF- β 2, TGF- β 3, TGF- β 4, or TGF- β 5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20 and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of any of the above-listed polypeptides.

Preferred antigens for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD46; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150.95, VLA-4, ICAM-1, VCAM, α 4/ β 7 integrin, and α v/ β 3 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-

CD11b antibodies); growth factors such as VEGF; tissue factor (TF); TGF- β alpha interferon (α -IFN); an interleukin, such as IL-8; IgE; blood group antigens Apo2, death receptor; flk2/flt3 receptor; obesity (OB) receptor; *mpl* receptor; CTLA-4; protein C etc. The most preferred targets herein are VEGF, IgE, TF, CD19, CD20, CD40, TGF- β ,

5 CD11a, CD18, Apo2 and C24.

Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these molecules (*e.g.* the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be
10 used as the immunogen. Such cells can be derived from a natural source (*e.g.* cancer cell lines) or maybe cells which have been transformed by recombinant techniques to express the transmembrane molecule. Other antigens and forms thereof useful for preparing antibodies will be apparent to those in the art.

The antibodies used as starting material in the methods of the present invention
15 maybe monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific to different epitopes of a single molecule or maybe specific to epitopes on different molecules. Methods for designing and making multispecific antibodies are known in the art. See, *e.g.*, Millstein *et al.* (1983) *Nature* 305:537-539; Kostelny *et al.* (1992) *J. Immunol.* 148:1547-1553; WO 93/17715.

20

Humanized Antibodies or Antigen Binding Fragments

The present invention is applicable to preparation of or modification of humanized antibodies or antigen binding fragment. Various methods for humanizing non-human
25 antibodies are known in the art. Preferably, a humanized antibody or antigen binding fragment has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "donor" residues, which are typically taken from an "donor" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.* (1986)
30 *Nature* 321:522-525; Riechmann *et al.* (1988) *Nature* 332:323-327; Verhoeyen *et al.* (1988) *Science* 239:1534-1536).

Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice,
35 humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Previous methods for preparing humanized antibodies have involved selecting human variable domains, both light and heavy, to be used in making the humanized antibodies. According to the so-called "best-fit" method, the entire sequence of the variable domain of rodent antibody is screened against the entire library of known human variable-
5 domain sequences. The human sequence which is closest to that of the rodent antibody is then accepted as the human framework for the humanized antibody (Sims et al. (1993) *J. Immunol.* 151:2296; Chothia et al. (1987) *J. Mol. Biol.* 196:901). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. Carter et al., (1992), *Proc. Natl. Acad. Sci.*
10 *USA* 89: 4285. In some cases, the framework region consensus sequences used are subgroup III for the heavy chain and subgroup I of the kappa light chain. These subgroups were the most commonly occurring subgroups based on the 1991 compilation of sequences of Kabat et al. cited supra.

The methods of the invention provide for improving the process of humanization by
15 identifying at least one human consensus subgroup FR sequence that can be combined with HVRs from a non-human monoclonal antibody to form a humanized antibody or antigen binding fragment that has improved characteristics for production in cell culture. In some embodiments, the method comprises expressing a variable domain comprising at least one FR sequence from a selected human subgroup variable domain consensus sequence, and a
20 HVR1 and/or HVR2 sequence of a non-human antibody in a host cell, wherein the selected human subgroup consensus sequence is the human subgroup consensus sequence that has a HVR1 and/or HVR2 sequence that has the most sequence identity to the HVR1 and/or HVR2 of the non-human antibody; and recovering the antibody variable domain from the host cell. The antibody variable domain can be a part of an antigen binding fragment, heavy
25 chain, light chain, or full length completely assembled antibody.

In other embodiments, the HVR1 and/ or HVR2 of the non-human monoclonal antibody are compared to the corresponding HVR1 and/or HVR2 sequences of the human subgroup consensus sequences, and the human consensus subgroup with the most sequence identity to the HVR1 and/or HVR2 of the non-human monoclonal antibody is selected to
30 provide at least one of the framework region sequences in the humanized antibody or antigen binding fragment. The variable domain can be a heavy or light chain or both. A single selected FR or more than one FR selected from the group consisting of FR1, FR2, FR3, FR4 and mixtures thereof can be used to prepare the recombinant antibody. In one embodiment, the recombinant antibody includes the selected FR1 of the human heavy chain
35 subgroup consensus sequence that has the most sequence identity with HVR1 and/or HVR2 of the non-human antibody. Identifying and selecting a human consensus subgroup to use for the source of at least one FR sequence may shorten the time required to form a

humanized antibody or antigen binding fragment that can be produced in high yield in cell culture. The host cells can be prokaryotic or eukaryotic.

In some embodiments, a method for preparing a humanized antibody or antigen binding fragment comprises expressing a variable domain in a host cell, wherein the variable domain has a substitution of at least one amino acid position proximal to a cys residue that participates in an intrachain variable domain disulfide bond with a different amino acid, wherein the different amino acid is the amino acid found at corresponding position of a human subgroup variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 amino acid sequence of the variable domain; and recovering the antibody variable domain from the host cell. The antibody variable domain can be a part of an antigen binding fragment, heavy chain, light chain, or full length completely assembled antibody.

In one embodiment, the method comprises comparing a HVR1 and/or HVR2 amino acid sequence of a variable domain of a non-human antibody to a corresponding HVR1 and/or HVR2 amino acid sequence of each human subgroup variable domain consensus sequences and selecting a human subgroup variable domain consensus sequence that has the most sequence identity with the HVR1 and/or HVR2 sequence of the variable domain; identifying at least one framework region to combine with the HVR1 and/HVR2 of the non-human antibody to form a variable domain of a humanized antibody or antigen binding fragment; further modifying the humanized antibody or antigen binding fragment by identifying at least one amino acid position in the variable domain of the humanized antibody or antigen binding fragment that is proximal to a cys residue that forms an intrachain variable domain disulfide bond in the variable domain; and placing at said at least one identified position in the variable domain the amino acid found at the corresponding position in the selected subgroup consensus sequence to form a modified variable domain of the humanized antibody or antigen binding fragment.

Methods for Improving the Yield of Antibodies: Modifying FRs

A method of the invention provides for modifying the framework regions of the variable domains of a parent antibody or antigen binding fragment to improve yield and/or designing a variable domain for improved yield when produced in cell culture. The parent antibody variable domain sequences for the method may be obtained from antibodies produced by various means. A naturally occurring antibody or synthetic antibody variable domain sequences can provide the framework to be modified. The method of the invention can also be employed during the process of humanization to select at least one framework region for use in the humanized antibody or antigen binding fragment.

In some embodiments, a method comprises expressing a variable domain of the antibody or antigen binding fragment comprising at least one modified FR in a host cell, wherein the modified FR has a substitution of at least one amino acid position in at least one FR with a different amino acid, wherein the different amino acid is the amino acid found at the corresponding FR position of a human subgroup variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 sequence of the variable domain, wherein the antibody or antigen binding fragment with the modified FR has improved yield in cell culture compared to an unmodified parent antibody or antigen binding fragment; and recovering the antibody or antigen binding fragment variable domain comprising the modified framework from the host cell. The antibody variable domain can be a part of an antigen binding fragment, heavy chain, light chain, or full length completely assembled antibody.

In some embodiments, a method comprises expressing a variable domain of the antibody or antigen binding fragment comprising at least one modified FR in a host cell, wherein the modified FR is obtained by substituting at least one amino acid in a FR of a parent variable domain of the antibody or antigen binding fragment with a different amino acid; wherein the different amino acid is an amino acid found at the corresponding FR position of a human subgroup variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 amino acid sequence of the parent variable domain to form a modified FR. The antibody or antigen binding fragment with the modified FR has improved yield in cell cultures compared to an antibody or antigen binding fragment comprising the parent variable domain. The antibody or antigen binding fragment variable domain comprising the modified framework is recovered from the host cell. The antibody variable domain can be a part of an antigen binding fragment, heavy chain, light chain, or full length completely assembled antibody.

In some embodiments of the invention, a method comprises, expressing a variable domain of the antibody or antigen binding fragment comprising at least one modified FR of a heavy chain in a host cell, wherein the modified FR has a substitution of at least one amino acid position in at least one FR with a different amino acid, wherein the different amino acid is the amino acid found at the corresponding FR position of a human subgroup heavy chain variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 sequence of the variable domain, wherein the antibody or antigen binding fragment with the modified FR of the heavy chain has improved yield in cell culture compared to an unmodified parent antibody or antigen binding fragment; and recovering the antibody or

antigen binding fragment variable domain comprising the modified framework from the host cell. The antibody variable domain can be a part of an antigen binding fragment, heavy chain, light chain, or full length completely assembled antibody.

In one embodiment of the invention, the method comprises a) comparing a HVR1 and/or HVR2 amino acid sequence of a variable domain or parent antibody or antigen binding fragment to a corresponding HVR1 and/or HVR2 amino acid sequence of each of human variable domain subgroup consensus amino acid sequences and selecting the subgroup consensus sequence that has the most sequence identity with the HVR1 and/or HVR2 amino acid sequence of the variable domain; b) identifying at least one amino acid in a FR in the variable domain that is different from an amino acid of the corresponding position of the selected subgroup consensus sequence; and c) substituting the at least one amino acid so identified with the corresponding amino acid residue of the selected subgroup consensus sequence to form a variable domain with modified framework region. The antibody or antigen binding fragment with the modified framework region has improved yield when produced in cell culture compared to the parent antibody or antigen binding fragment from which it was derived.

In some embodiments, at least two or more of the residues in a FR of the variable domain are substituted with the corresponding amino acid residues of the selected subgroup consensus sequence, and preferably, all of the residues that are different in the FR are substituted with the corresponding amino acids from the selected subgroup consensus sequence. In one embodiment, all of the residues that are different in FR1 are substituted with the corresponding amino acids from the selected subgroup consensus sequences

The parent antibody or antigen binding fragment provides variable domain sequences as source materials for the methods of the invention. Variable domain sequences may be known or readily ascertained using known methods. A parent antibody or antigen binding fragment is modified in accord with the methods of the invention to improve yield when produced in cell culture. Parent antibodies can include humanized antibody, a chimeric antibody, a monoclonal antibody, a human antibody, a multispecific antibody, diabodies, or an antibody generated by phage display. Antigen binding fragments can include Fab fragments, F(ab')₂ fragments, scFv fragments, scFv2 fragments, a single arm antibody comprising one light chain, one heavy chain and a N- terminally truncated heavy chain constant region sufficient to form a Fc region capable of increasing the half life of the single arm antigen binding molecule, or single chain antibodies.

A HVR1 and/or HVR2 amino acid sequence of a variable domain of parent antibody or antigen binding fragment is identified and compared to the corresponding HVR1 and/or HVR2 amino acid sequence of each human variable domain subgroup consensus sequences. Preferably, the HVR1 sequences are compared. A HVR1 and/or

HVR2 amino acid sequence can be obtained from a heavy or light chain variable domain or both. The HVR1 and/or HVR2 includes amino acids from a CDR and/or in a hypervariable loop or both. For example, in the case of some humanized antibodies, the HC-HVR1 includes amino acids of both the CDR and hypervariable loop, i.e. amino acids 26-35.

5 Once the amino acid sequence of a HVR1 and/or HVR2 of a variable domain is identified, it is aligned with the amino acid sequence at the corresponding positions in each of the human variable domain subgroup consensus sequences. A consensus sequence for each variable domain subgroup is derived by selecting the most frequently occurring amino acid at each position in the known sequences of variable domains. The sequences of human
10 variable domains from naturally occurring antibodies have been and continue to be compiled online and/ or in published form. A useful compilation of sequences of naturally occurring immunoglobulins is that prepared by Kabat; Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institute of Health, Bethesda, MD (1991) and published online at immuno-bme-nwu-edu. Subgroups of the
15 variable domains of naturally occurring antibodies have been identified based on overall sequence similarity. For example, Kabat has identified 3 subgroups for the human heavy chain variable domain: Subgroup I, Subgroup II and Subgroup III. Kabat et al. *supra*. The consensus sequence for a subgroup of variable domains may change as more sequences of naturally occurring antibodies are added to the subgroup.

20 After aligning the HVR1 and/or HVR2 sequences, the subgroup consensus sequence with the most sequence identity to the HVR1 and/or HVR2 amino acid sequence of the variable domain that is to be modified is selected. Once a subgroup consensus sequence is selected, a FR sequence of the variable domain is compared to the corresponding FR sequence in the selected subgroup consensus sequence. The amino acid
25 positions in the FR where the two sequences differ are identified and at least one amino acid position in the FR that differs is substituted with the amino acid at that position in the selected human subgroup consensus sequence. In some embodiments, at least about 10%, 30%, 50%, 70%, 80%, 90% of the amino acid positions that differ are subjected to substitution as described.

30 In one embodiment, a FR sequence of a variable domain is obtained from a heavy chain, a light chain or both. One or more FRs selected from the group consisting of FR1, FR2, FR3, FR4 and mixtures thereof can be substituted at at least one amino acid residue that differs from the selected subgroup consensus sequence. A FR can be modified at one or more than one amino acid residues. In some embodiments, at least two amino acid positions
35 in at least one FR are substituted with amino acids in the corresponding positions of the selected subgroup sequence. In some embodiments, the FR is a heavy chain FR1 and one of the identified amino acid positions is position 6 or position 23 or both, and the other position

is selected from the group consisting of amino acid positions 1, 11, 13, 18, 19, and mixtures thereof. In some embodiments, all of the amino acid positions at positions 1, 6, 11, 13, 18, 19, and 23 of the heavy chain FR1 are substituted.

A FR of the variable domain of a parent antibody or antigen binding fragment can be modified such that it has any one of at least 50% to 100% sequence identity to the corresponding FR sequence of the selected subgroup consensus sequence, preferably at least 50%, more preferably at least 55%, more preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%, identical in sequence to the corresponding FR sequence of the selected subgroup consensus sequence.

The HVR1 of a heavy or light chain can be compared to the corresponding HVR1 sequences of the human heavy or light chain subgroup consensus sequences. In one embodiment, the heavy chain HVR1 of anti-VEGF humanized antibody VNERK (amino acids 26 to 35) is compared to the corresponding HVR1 sequences of the human heavy chain subgroup consensus sequences:

Sequence	HVR Alignment	% Identity
Subgroup I HVRH1:	GYTFTSYAIS (SEQ ID NO: 15)	70%
VNERK HVR1:	 GYTFTNYGIN (SEQ ID NO: 14)	7/10
Subgroup II HVRH1:	GGSVSSYWSN (SEQ ID NO: 16)	18%
VNERK HVR1:	 GYTFTNYGIN. (SEQ ID NO: 14)	2/11
Subgroup III HVRH1:	GFTFSSYAMS (SEQ ID NO: 17)	40%
VNERK HVR1:	 GYTFTNYGIN (SEQ ID NO: 14)	4/10

The human consensus subgroup having the most sequence identity with HVR1 is the subgroup I sequence and at least one of the FR sequences of the consensus subgroup sequence I is compared to the corresponding FR sequence of the of the anti-VEGF antibody. Those positions that differ between the two sequences are selected for substitutions.

In other embodiments, The HVR2 of a heavy or light chain can be compared to the corresponding HVR2 sequences of the human heavy or light chain subgroup consensus sequences. In one embodiment, the Kabat defined heavy chain HVR2 of anti-VEGF humanized antibody VNERK (amino acids 50 to 65) is compared to the corresponding HVR2 sequences of the human heavy chain subgroup consensus sequences. The human consensus subgroup having the most sequence identity with HVR2 is the subgroup I sequence and at least one of the FR sequences of the consensus subgroup sequence I is

compared to the corresponding FR sequence of the of the anti-VEGF antibody. Those positions that differ between the two sequences are selected for substitutions.

In other embodiments, the HVR1 and the HVR2 of a heavy or light chain can be compared to the corresponding HVR1 and HVR2 sequences of the human heavy or light chain subgroup consensus sequences. If the human consensus subgroup having the most sequence identity with HVR1 differs from that of the HVR2 sequence, then the human consensus subgroup having the most sequence identity with HVR1 sequence is selected. For example, in one embodiment, the heavy chain HVR1 of anti-VEGF humanized antibody VNERK (amino acids 26 to 35) is compared to the corresponding HVR1 sequences of the human heavy chain subgroup consensus sequences and the Kabat defined heavy chain HVR2 of anti-VEGF humanized antibody VNERK (amino acids 50 to 65) is compared to the corresponding HVR2 sequences of the human heavy chain subgroup consensus sequences. The human consensus subgroup having the most sequence identity with HVR1 and HVR2 is the subgroup I sequence and at least one of the FR sequences of the consensus subgroup sequence I is compared to the corresponding FR sequence of the of the anti-VEGF antibody. Those positions that differ between the two sequences are selected for substitutions.

In an anti-VEGF antibody (described herein) comprising amino acid subgroup III consensus sequence in the heavy chain FR1, substitution of the FR1 subgroup III consensus sequence with the corresponding amino acid residues of the selected human consensus subgroup I in the heavy chain FR1 resulted in improved antibody yield. Conversion of the heavy chain subgroup III FR1 residues of this antibody to the subgroup I residues required 12 amino acid substitutions in the FR1 sequence. The modified FR sequence with all 12 substitutions has 100% sequence identity to the selected human subgroup consensus sequence. A single amino acid substitution at a consensus sequence III residue with a consensus sequence subgroup I residue results in a modified FR sequence that has about 56% sequence identity with the selected subgroup consensus sequence.

When the antibody to be modified in accord with the methods of the invention is a humanized antibody or antigen binding fragment, some changes to the framework and HVRs for improving binding affinity may have been made during the process of humanization. For example, the humanized anti-VEGF VNERK antibody was prepared with heavy chain consensus framework region sequences from subgroup III. In the process of improving antibody binding affinity, seven changes were made in the framework region sequences. When the changes at these positions were made during the process of humanization, the amino acids substituted at each of these positions was that of the murine antibody at the corresponding position. Four of the changes also happen to correspond to a change from a subgroup consensus subgroup III residue to a subgroup I residue and one

represents a conservative substitution for the subgroup I sequence. Because the humanized antibody or antigen binding fragment may have some of the FR region substitutions at the positions identified in accord with the methods of the invention, the improvement of the yield may be less than that would be expected if the changes to the subgroup III sequence had not already been made to the humanized antibody. Producing the anti-VEGF VNERK antibody with heavy chain FR regions from the human consensus subgroup I instead of subgroup III initially using the methods of the invention could have shortened the time to producing an antibody that can be produced in high yield in cell culture.

In another embodiment, at least one amino acid, and preferably all, of the amino acids in positions in the FR1 of the heavy chain variable domain that differ from the selected subgroup consensus sequence are substituted with subgroup consensus amino acids at those positions. For example, a humanized antibody prepared with framework regions from heavy chain variable domain subgroup III consensus sequence can be modified as follows. The FR1 of the heavy chain variable domain can be replaced either with the FR1 sequence of the subgroup I or subgroup II consensus sequences depending on which subgroup sequence has the most sequence identity with the HVR1 and/or HVR2 sequence of the heavy chain variable domain. The human heavy chain FR1 subgroup consensus sequence can be selected from the group consisting of SEQ. ID NO:1, SEQ ID NO:2 and SEQ ID NO:3. As illustrated in the examples, the FR1 sequence of an anti-VEGF antibody comprising the heavy chain subgroup III consensus FR1 sequence can be replaced with the heavy chain subgroup I consensus FR1 by making a total of 12 amino acid substitutions at those amino acid residues that differ between the two sequences.

The antibodies or antigen binding fragments with at least one modified framework region amino acid formed in accord with the process of the invention are characterized by improved yield of assembled antibody products when produced in cell culture under similar conditions and cell types as the unmodified antibodies or antigen binding fragments. The host cells can be prokaryotic or eukaryotic. In some embodiments, the antibodies or antigen binding fragments with modified framework regions are produced in high yield without detrimental effects on expression levels or on antigen binding affinity. In some cases, further modifications to the modified antibody may be necessary, for example, to enhance antigen binding affinity. For example, when the methods of the invention are applied to producing a humanized antibody or antigen binding fragment, other changes to antibody sequence may be made to improve antigen binding affinity. If improvement in antigen binding affinity of antibodies or antigen binding fragments with modified framework regions is necessary, it can be achieved by altering residues as needed and in accord with methods well-known in the art (*i.e.*, Presta et al., *supra*) and as described herein.

The relative yield can be determined using standard methods such as by separating out the antibody or antigen binding fragment produced in cell culture by SDS-PAGE, transferring the protein to an immunoblot, and analyzing the amount of completely assembled antibody products by scanning densitometry. The yield of the completely assembled antibody or antigen binding fragment is improved at least about 2 fold or more, more preferably about 2 to 4 fold, more preferably about 2 to 8 fold, and most preferably about 2 to 16 fold when compared to the yield of unmodified antibody or antigen binding fragment when produced under similar cell culture conditions.

In addition, the method for improved yield of antibodies can be applied to producing a humanized antibody or antigen binding fragment for improved folding and/or yield. The method can be applied to the humanization process and may result in decreasing the time it takes to prepare a humanized antibody with the desired characteristics. Once a HVR1 and/or HVR2 amino acid sequence of a variable domain is selected, the HVR1 and/or HVR2 sequence can be aligned and compared to the corresponding sequence of each of the human subgroup consensus sequences for that variable domain and the subgroup with the most identity to HVR1 and/or HVR2 sequence is selected. The selected subgroup sequence is then used to provide at least one FR sequence for the antibody or antigen binding fragment that is being produced.

The invention also includes antibody variable domains, antigen binding fragments, heavy chains, light chains, and/or full length completely assembled antibodies produced or modified in accord with the methods of the invention.

Methods for Improving Antibody Yield: Modifying Residues Proximal to a Cys Residue that Forms an Intrachain Disulfide Bond.

Another aspect of the invention includes a method for preparing a humanized antibody or antigen binding fragment or for improving the yield of antibodies or antigen binding fragments when produced in cell culture by modifying at least one amino acid residue in a variable domain proximal to a cys residue that forms an intrachain variable domain disulfide bond. The at least one amino acid position is modified by placing in that position a different amino acid, wherein the different amino acid is found at the corresponding position in a human variable domain subgroup consensus sequence that has the most sequence identity with a HVR1 and/or HVR2 amino acid sequence of the variable domain. Modifications can be made to either the heavy chain or light chain variable domain. The antibody variable domain so modified can be a part of an antigen binding fragment, heavy chain, light chain, or full length completely assembled antibody. In one embodiment, at least two positions are modified: either in a single variable domain or one substitution in each of the heavy and light chain variable domains.

In some embodiments, the method comprises expressing a modified variable domain of the antibody or antigen binding fragment in a host cell, wherein the modified variable domain has a substitution of at least one amino acid position proximal to a cys residue that participates in an intrachain variable domain disulfide bond with a different amino acid, wherein the different amino acid is the amino acid found at corresponding position of a human subgroup variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 amino acid sequence of the variable domain, wherein the antibody or antigen binding fragment comprising the modified variable domain has improved yield in cell culture compared to the antibody or antigen binding fragment; and recovering the antibody or antigen binding fragment comprising the modified variable domain from the host cell.

A variable domain is modified at one or more amino acid positions that are proximal to a cys residue that forms an intrachain variable domain disulfide bond. Typically, each variable domain has a single intrachain disulfide bond between 2 cys residues. The position of cys residues that form the intrachain disulfide bond in a variable domain are usually at conserved positions *e.g.* at positions L23 and L88 in the light chain and at positions H22 and H92 in the heavy chain. Amino acid positions proximal to a cys residue are those positions that are near to the cys residue in a three-dimensional structure of the antibody or adjacent to the cys residue in a linear sequence. A position adjacent to the cys residue includes the two amino acid positions on either side of the cys residue in a linear sequence. A position is near to a cys residue in a three-dimensional structure if the side chain of the amino acid (or the α carbon for Gly) in that position is about 5 angstroms or less from the cys residue or is a position where the amino acid in that position has lost about 10 square angstroms or more of solvent accessible surface area by contacting the cys residue.

One way to identify amino acid positions proximal to cys residues in a three-dimensional structure involves analysis of the crystal structure of the antibody or antigen binding fragment using a computer program such as MIDAS (available from University of California San Francisco). To determine amino acid positions proximal to cys residues based on a loss of surface area, computer programs such as SOLV may be employed. If a crystal structure of the antibody or antigen binding fragment is not available, a three-dimensional structure based on the primary sequence may be modeled. Computer programs are available which illustrate probable three-dimensional conformational structures of antibody sequences.

For antibodies that have the framework regions from the same human subgroup consensus sequence, amino acid positions proximal to the cys residues may be conserved. For example, for antibodies that have FRs in the heavy chain with the sequence of the human heavy chain variable domain subgroup III consensus sequence, the amino acid

positions identified as proximal to the cys residues in the three dimensional structure of the heavy chain include position 4, position 6, position 34, position 36, position 78, and position 104 (numbering according to Kabat et al., supra). For antibodies that have FRs in the light chain with the sequence of human light chain variable domain Kappa subgroup I
5 consensus sequence, the amino acid positions identified as proximal to cys residues in the three dimensional structure of the light chain include position 4, position 6, position 33, position 35, and position 71.

Proximal residues adjacent to a cys residue can include residues in positions adjacent to the cys residues in the linear sequence. The position of cys residues that form the
10 intrachain disulfide bond in a variable domain are usually at conserved positions e.g. at positions L23 and L88 in the light chain and at positions H22 and H92 in the heavy chain. In the heavy chain variable domain, amino acid positions adjacent to the cys residues include amino acid positions 20, 21, 23, 24, 90, 91, 93, and 94. Proximal residues adjacent to a cys residue in the light chain variable domain include residues at amino acid positions 21, 22,
15 24, 25, 86, 87, 89 and 90.

Once at least one amino acid position proximal to the cys residue is identified, an amino acid from the corresponding position in the human variable domain subgroup consensus sequence with the most sequence identity to the HVR1 and/or HVR2 amino acid sequence of the variable domain is placed at that position if the amino acid at that position is
20 different from the amino acid in the variable domain. As described previously, the human variable domain subgroup consensus sequence with the most identity is selected after the HVR1 and/or HVR2 sequence of the variable domain of the antibody or antigen binding fragment is aligned and compared with the corresponding sequence of each of subgroup consensus sequences. An amino acid is placed at a position proximal to the cys residue,
25 typically by substituting the amino acid residue at that position with corresponding amino acid from the selected subgroup consensus sequence.

In some embodiments, at least two positions proximal to a cys residue are modified. The two positions can be modified in a single variable domain or two positions can be modified by making a single substitution in a light chain variable domain and a single
30 substitution in a heavy chain variable domain. In some embodiments, a heavy chain variable domain has a substitution at amino acid position 4, 6, 34, 78, or mixtures thereof. In other embodiments, a light chain variable domain has a substitution at amino acid positions 4, 71, or mixtures thereof. In some embodiments, a heavy chain has substitutions at position 6 and position 34, and a light chain has a substitution at position 4 and at position
35 71. In some embodiments, all of the amino acid positions proximal to a cys residue in a heavy or light chain are substituted.

The antibodies or antigen binding fragments with at least one modification to at least one amino acid proximal to a cys residue formed in accord with process of the invention are characterized by improved yield of assembled antibody or antigen binding fragment when produced in cell culture. Preferably, the antibodies or antigen binding fragments have improved yield with minimal, if any, detrimental effect on expression levels, the antigen binding affinity, biological activity and/or physicochemical properties. If improvement in antigen binding affinity of antibodies with modified framework regions is needed it can be achieved by altering HVR residues as needed and in accord with methods known in the art, for example as described by Presta et al, supra. The relative yield can be determined using standard methods such as by separating out the antibody product produced in cell culture by SDS-PAGE, transferring the protein to an immunoblot, and analyzing the amount of assembled antibody products by scanning densitometry. The yield of assembled antibody products is improved at least about 2 fold or more, more preferably, about 2 to 4 fold, about 2 to 8 fold and most preferably about 2 to 16 fold when compared to the yield of unmodified antibody or antigen binding fragment when produced under similar cell culture conditions.

In addition, the method for improving the yield of antibodies can be applied to producing an antibody or antigen binding fragment, for example, a humanized antibody or antigen binding fragment, for improved folding and yield. Once a HVR1 and/or HVR2 region sequence of a variable domain is selected, the HVR1 and/or HVR2 sequence can be aligned and compared to the corresponding sequence of each of the human subgroup consensus sequences and the subgroup with the most identity to HVR1 and/or HVR2 sequence selected. The selected subgroup sequence is then used to identify an amino acid(s) to place in at least one amino acid position, preferably all of the positions, proximal to the cys residues that form an intrachain disulfide bond for the antibody or antigen binding fragment in the humanized antibody or antigen binding fragment.

The invention also includes antibody variable domains, antigen binding fragments, heavy chains, light chains, and/or full length completely assembled antibodies produced or modified in accord with the methods of the invention.

30

Methods for Preparing Antibodies or Antigen Binding Fragments with Modified Framework Region Residues

When the methods of the invention are applied to antibodies or antigen binding fragments whose sequences are known or readily ascertainable using known methods, targeted substitutions are made in at least one FR residue and/or in at least one amino acid position proximal to cys residues that form an intrachain disulfide bond. The antibodies

with amino acid substitutions in the variable domain can be prepared synthetically or by using recombinant methods.

For recombinant methods, nucleic acid sequences encoding variable domains of heavy and light chains are prepared using standard methods. The sequences of the variable domains of many naturally occurring antibodies and the human subgroup consensus sequences are known and sequences of humanized or antigen binding fragments can be readily determined by standard methods. Nucleic acid molecules encoding variable domains with at least one modification in the framework region and/or with substitutions at one or more positions proximal to cys residues are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants), synthesis, or preparation by oligonucleotide-mediated mutagenesis, site-directed mutagenesis, PCR mutagenesis, and cassette mutagenesis of a parent variable domain of an antibody or antigen binding fragment.

Using recombinant methods, a polynucleotide molecule encoding the heavy and light chain variable domain of the antibody is modified using standard methods. For example, the polynucleotide molecule encoding the heavy and/or light chain variable domain can be modified in a single step using a double stranded oligonucleotide encoding one or more amino acid substitutions according to the method described by Carter et al., *PNAS* 89:4285 (1992) or as described in U.S. Patent No. 5,747,662.

The variable domains of the invention have one or more amino acid substitutions in FR residues and/or in positions proximal to cys residues of the variable domain. A FR of the variable domain of a parent antibody or antigen binding fragment can be modified such that it has any one of 50% to 100% sequence identity to the corresponding FR sequence of the selected subgroup consensus sequence, preferably at least 50%, more preferably at least 55%, more preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%, identical in sequence to the corresponding FR sequence of the selected subgroup consensus sequence.

A variable domain of a parent antibody or antigen binding fragment can be modified so that it has any one of 50% to 100% sequence identity to the corresponding variable domain of the parent antibody, preferably at least 50%, more preferably at least 55%, more preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%, identical in sequence to the corresponding variable domain sequence of the parent antibody or antigen binding fragment.

When an entire framework region is substituted at all of the residues that differ from the selected human consensus subgroup, more than one amino acid substitution may be made in the framework region to form a modified framework region that can be used to construct a variable domain or replace the framework region in a variable domain of a parent antibody or antigen binding fragment that has the HVR1 and/or HVR2 amino acid sequence. When more than one FR selected from the group consisting of FR1, FR2, FR3 and FR4 regions are modified to correspond to the sequence for the selected human consensus sequence, multiple amino acid substitutions may be made to form modified framework regions that can be used to replace framework regions in antibodies or antigen binding fragments with the selected HVR1 and/or HVR2 sequence or to construct a new variable domain.

Once variable domains are modified or prepared in accord with the methods of the invention, they can be combined with the appropriate constant domains to form a full length heavy or light chain using methods known to those of skill in the art. DNA constructs encoding the heavy and light chains can be coexpressed in host cells for production of completely assembled full length antibodies. The modifications can be in either the heavy or light chain variable domains or both.

Additional Modifications

In another aspect of the invention, antibodies or antigen binding fragments with additional amino acid sequence modification(s) are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody besides improved yield. These modifications may be made at amino acid positions other than at amino acid positions proximal to a cys residue that participates in an intrachain variable domain disulfide bond or at amino acid positions in one or more of the framework regions that have been identified and modified in accord with the process of the invention.

Another type of amino acid sequence modification is amino acid substitution at residues other than the selected FR residues or other than at amino acid positions proximal to cys residues that participate in an intrachain variable domain disulfide bond. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated, especially those that improve binding affinity. When the methods of the invention are used to produce a humanized antibody or antigen binding fragment, it may be desirable to make further modification to the antibody sequence after at least one FR has been selected in accord with the methods of the invention, for example, to enhance affinity. When the methods of the invention are applied to humanized antibodies, the human consensus framework sequences may have been modified to improve antigen binding affinity in the process of humanization. Some of those modifications may occur at

amino acid positions other than the selected FR residues such as at FR positions that have amino acids that do not differ between the selected subgroup consensus sequence and the parent variable domain FR sequence.

Conservative substitutions are shown in Table 1 under the heading of “preferred substitutions”. If such substitutions result in a change in biological activity, then more substantial changes, denominated “exemplary substitutions” as shown in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened, for example for improved binding affinity.

10

TABLE 1

Original Residue	Exemplary Substitutions	Exemplary Substitutions
Ala (A)	val; leu; lie	val
Arg (R)	lye; gln; asn	lys
Asn (N)	gln; his; asp; lys; arg	gln
Asp (D)	glu; asn	glu
Cys (C)	ser; ala	ser
Gln (Q)	asn; glu	asn
Gly (G)	ala	ala
His (H)	asn; gln; lys; arg	arg
Ile	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	tyr
Pro (P)	ala	ala
Ser (S)	thr; cys	cys
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

- (1) hydrophobic: norleucine, met, ala, val, leu, ile;
- (2) neutral hydrophilic: cys, ser, thr;
- (3) acidic: asp, glu;
- (4) basic: asn, gln, his, lys, arg;
- (5) residues that influence chain orientation: gly, pro; and
- (6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the antibody or not involved in forming the intrachain variable region disulfide bond also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking.

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g. a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated, especially antigen binding affinity. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (e.g. 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibodies thus generated are displayed from filamentous phage particles as fusions to a gene product of M13 (e.g. gene III) packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g. binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

It also may be desirable to introduce one or more amino acid modifications in an Fc region of the antibody of the invention, thereby generating a Fc region variant. The Fc region variant may comprise a human Fc region sequence (e.g., a human IgG1, 1gG2, 1gG3 or 1gG4 Fc region) comprising an amino acid modification (e.g. a substitution) at one or more amino acid positions.

These modifications to the amino acid sequence of the antibody or antigen binding fragment are prepared by introducing appropriate nucleotide changes into the antibody nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics of improved yield when produced in cell culture and antigen binding

specificity. The amino acid alterations may be introduced in the subject antibody amino acid sequence at the time that sequence is made.

Antibody or Antigen Binding Fragment Conjugates

5 Another aspect of the invention contemplates that antibodies or antigen binding fragments with a modified framework region are conjugated to another molecule. The other molecules can include a detectable label, a purification tag, another polypeptide (for e.g., a cytotoxic polypeptide) or a cytotoxic compound.

 Amino acid sequence conjugates include amino- and/or carboxyl-terminal fusions
10 ranging in length from one residue to polypeptides containing a hundred or more residues. Examples of terminal fusions or conjugates include an antibody with an N-terminal methionyl residue of the antibody fused to a cytotoxic polypeptide or compound. Other fusions of the antibody molecule include the fusion of the N- or C-terminus of the antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the
15 antibody.

Vector Construction

 Polynucleotide sequences encoding the immunoglobulin light and heavy chains of the invention can be obtained using standard recombinant techniques. Desired
20 polynucleotide sequences may be isolated and sequenced from antibody producing cells such as hybridoma cells. Alternatively, polynucleotides can be synthesized using nucleotide synthesizer or PCR techniques. Once obtained, sequences encoding the light and heavy chains are inserted into a recombinant vector capable of replicating and expressing heterologous polynucleotides in host cells. Many vectors that are available and known in
25 the art can be used for the purpose of the present invention. Selection of an appropriate vector will depend mainly on the size of the nucleic acids to be inserted into the vector and the particular host cell to be transformed with the vector. Each vector contains various components, depending on its function (amplification or expression of heterologous polynucleotide, or both) and its compatibility with the particular host cell in which it resides.
30 The vector components generally include, but are not limited to: an origin of replication, a selection marker gene, a promoter, a ribosome binding site (RBS), a signal sequence, the heterologous nucleic acid insert and a transcription termination sequence.

 In general, plasmid vectors containing replicon and control sequences that are derived from species compatible with the host cell are used in connection with these hosts.
35 The vector ordinarily carries a replication site, as well as marking sequences that are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species. pBR322 contains

genes encoding ampicillin (Amp) and tetracycline (Tet) resistance and thus provides easy means for identifying transformed cells. pBR322, its derivatives, or other microbial plasmids or bacteriophage may also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of endogenous proteins. Examples of
5 pBR322 derivatives used for expression of particular antibodies are described in detail in Carter et al., U.S. Patent No. 5,648,237, and the "Examples" section herein below.

In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as transforming vectors in connection with these hosts. For example, bacteriophage such as λ GEM.TM.-11 may be utilized in
10 making a recombinant vector which can be used to transform susceptible host cells such as *E. coli* LE392.

An expression vector for prokaryotic host cells comprises a promoter-cistron pair or multiple pairs. The promoter is an untranslated regulatory sequence located upstream (5') to a cistron that modulates the cistron's expression. Prokaryotic promoters typically fall into
15 two classes, inducible and constitutive. Inducible promoter is a promoter that initiates increased levels of transcription of the cistron under its control in response to changes in the culture condition, e.g. the presence or absence of a nutrient or a change in temperature. Although both constitutive and inducible promoters can be used in the present invention, inducible promoters under tight regulation are preferred in the prokaryotic expression
20 vectors disclosed herein. A large number of promoters recognized by a variety of potential host cells are well known. The selected promoter can be operably linked to cistron DNA encoding the light or heavy chain by removing the promoter from the source DNA via restriction enzyme digestion and inserting the isolated promoter sequence into the vector of the invention. Both the native promoter sequence and many heterologous promoters may be
25 used to direct amplification and/or expression of the target genes. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed target gene as compared to the native target polypeptide promoter.

Promoters suitable for use with prokaryotic hosts include the PhoA promoter, the β -galactosidase and lactose promoter systems, a tryptophan (*trp*) promoter system, T7
30 promoter, and hybrid promoters such as the *tac*, *tacII* or the *trc* promoter. However, other promoters that are functional in bacteria (such as other known bacterial or phage promoters) are suitable as well. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to cistrons encoding the target light and heavy chains (Siebenlist et al. (1980) *Cell* 20: 269) using linkers or adaptors to supply any required
35 restriction sites. More preferred promoter for use in this invention is the PhoA promoter.

For recombinant production of the antibody, the nucleic acid encoding it may be isolated and inserted into a replicable vector for further cloning (amplification of the DNA)

or for expression in cells, for example eukaryotic cells. DNA encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components
5 generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, e.g., as described in U.S. Pat. No. 5,534,615 issued Jul. 9, 1996 and specifically incorporated herein by reference.

In one aspect of the present invention, each cistron within the recombinant vector
10 comprises a secretion signal sequence component that directs translocation of the expressed polypeptides across a membrane. In general, the signal sequence maybe a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector. The signal sequence selected for the purpose of this invention should be one that is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell. For
15 prokaryotic host cells that do not recognize and process the signal sequences native to the heterologous polypeptides, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group consisting of the alkaline phosphatase, penicillinase, or heat-stable enterotoxin II (STII) leaders, LamB, PhoE, PelB, OmpA and MBP. In a preferred embodiment of the invention, the signal sequences used in both
20 cistrons of the expression system are STII signal sequences or variants thereof.

In another aspect, the production of the antibodies or antigen binding fragments according to the invention can occur in the cytoplasm of the host cell, and therefore does not require the presence of secretion signal sequences within each cistron. In that regard, immunoglobulin light and heavy chains are expressed, folded and assembled to form
25 functional immunoglobulins within the cytoplasm. Certain host strains (e.g., the *E. coli* *trxB*⁻ strains) provide cytoplasm conditions that are favorable for disulfide bond formation, thereby permitting proper folding and assembly of expressed protein subunits. Probe and Pluckthun, *Gene*, 159:203 (1995).

The use of an expression system in which the quantitative ratio of expressed light
30 and heavy chains can be modulated in order to maximize the yield of secreted and completely assembled antibodies or antigen binding fragments is preferred. Such modulation is accomplished by simultaneously modulating translational strengths for light and heavy chains. One technique for modulating translational strength is disclosed in Simmons et al. U.S. Pat. No. 5,840,523.

35 Preferably, a set of vectors is generated with a range of TIR strengths for each cistron therein. This limited set provides a comparison of expression levels of each chain as well as the yield of assembled products under various TIR strength combinations. TIR

strengths can be determined by quantifying the expression level of a reporter gene as described in detail in Simmons et al. U.S. Pat. No. 5,840,523. For the purpose of this invention, the translational strength combination for a particular pair of TIRs within a vector is represented by (N-light, M-heavy), wherein N is the relative TIR strength of light chain and M is the relative TIR strength of heavy chain. For example, (3-light, 7-heavy) means the vector provides a relative TIR strength of about 3 for light chain expression and a relative TIR strength of about 7 for heavy chain expression. Based on the translational strength comparison, the desired individual TIRs are selected to be combined in the expression vector constructs of the invention.

Prokaryotic host cells suitable for expressing antibodies of the invention include Archaeobacteria and Eubacteria, such as Gram-negative or Gram-positive organisms. Examples of useful bacteria include Escherichia (e.g., *E. coli*), Bacilli (e.g., *B. subtilis*), Enterobacteria, Pseudomonas species (e.g., *P. aeruginosa*), Salmonella typhimurium, Serratia marcescens, Klebsiella, Proteus, Shigella, Rhizobia, Vitreoscilla, or Paracoccus. Preferably, gram-negative cells are used. More preferably, *E. coli* cells are used as hosts for the invention. Preferred *E. coli* strain are strain W 3110 (Bachmann, *Cellular and Molecular Biology*, vol. 2 (Washington, D.C.: American Society for Microbiology, 1987), pp. 1190-1219; ATCC Deposit No. 27,325) and derivatives thereof, including strain 33D3 having genotype W 3110 *kan^R ΔfhuA (Δ tonA) ptr3 lacIq lacL8 ompTΔ(nmpc-fepE) deg P* (U.S. Pat. No. 5,639,635). Of course other strains and derivatives thereof, such as *E. coli* 294 (ATCC 31,446), *E. coli* B, *E. coli* 1776 (ATCC 31,537) and *E. coli* RV308 (ATCC 31,608) are also suitable. These examples are illustrative rather than limiting. Methods for constructing derivatives of any of the above-mentioned bacteria having defined genotypes are known in the art and described in, for example, Bass et al., *Proteins*, 8:309-314 (1990). It is, of course, necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, *E. coli*, Serratia, or Salmonella species can be suitably used as the host when well known plasmids such as pBR322, pBR325, pACYC177, pUC or pKN4IO are used to supply the replicon. Preferably the host cell should secrete minimal amounts of proteolytic enzymes, and additional protease inhibitors may desirably be incorporated in the cell culture.

Other suitable host cells are described below. In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for vectors encoding a modified polypeptide. *Saccharomyces cerevisiae* is a commonly used lower eukaryotic host microorganism. Others include *Schizosaccharomyces pombe* (Beach and Nurse, *Nature*, 290: 140 [1981]; EP 139,383 published 2 May 1985); *Kluyveromyces* hosts (U.S. Patent No. 4,943,529; Fleer et al., *Bio/Technology*, 9:968-975 (1991)) such as, e.g., *K. lactis* (MW98-8C, CBS683, CBS4574; Louvencourt et al., *J. Bacteriol.*, 154(2):737-

742 [1983]], *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickerhamii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906; Van den Berg et al., *Bio/Technology*, 8:135 (1990)), *K. thermotolerans*, and *K. marxianus*; *yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070; Sreekrishna et al., *J. Basic Microbiol.*, 28:265-278 [1988]); *Candida*; *Trichoderma reesia* (EP 244,234); *Neurospora crassa* (Case et al., *Proc. Natl. Acad. Sci. USA*, 76:5259-5263 [1979]); *Schwanniomyces* such as *Schwanniomyces occidentalis* (EP 394,538 published 31 October 1990); and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium* (WO 91/00357 published 10 January 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance et al., *Biochem. Biophys. Res. Commun.*, 112:284-289 [1983]; Tilburn et al., *Gene*, 26:205-221 [1983]; Yelton et al., *Proc. Natl. Acad. Sci. USA*, 81: 1470-1474 [1984]) and *A. niger* (Kelly and Hynes, *EMBO J.*, 4:475-479 [1985]). Methylophilic yeasts are suitable herein and include, but are not limited to, yeast capable of growth on methanol selected from the genera consisting of *Hansenula*, *Candida*, *Kloeckera*, *Pichia*, *Saccharomyces*, *Torulopsis*, and *Rhodotorula*. A list of specific species that are exemplary of this class of yeasts may be found in C. Anthony, *The Biochemistry of Methylophilic Yeasts*, 269 (1982).

Suitable host cells for the expression of modified antibodies are derived from multicellular organisms. Examples of invertebrate cells include insect cells such as *Drosophila* S2 and *Spodoptera* Sf9, as well as plants and plant cells.

Examples of useful mammalian host cell lines include Chinese hamster ovary (CHO) and COS cells. More specific examples include monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.*, 23:243-251 (1980)); and mouse mammary tumor (MMT 060562, ATCC CCL51).

Antibody Production

Host cells are transformed with the above-described expression vectors and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transformation means introducing DNA into the prokaryotic host so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride is generally used for bacterial cells that contain substantial cell-wall barriers. Another method for transformation employs polyethylene glycol/DMSO. Yet another technique used is electroporation.

Prokaryotic cells used to produce the polypeptides of the invention are grown in media known in the art and suitable for culture of the selected host cells. Examples of suitable media include Luria-Bertani (LB) broth plus necessary nutrient supplements. In preferred embodiments, the media also contains a selection agent, chosen based on the construction of the expression vector, to selectively permit growth of prokaryotic cells containing the expression vector. For example, ampicillin is added to media for growth of cells expressing ampicillin resistant gene.

Any necessary supplements besides carbon, nitrogen, and inorganic phosphate sources may also be included at appropriate concentrations introduced alone or as a mixture with another supplement or medium such as a complex nitrogen source. Optionally, the culture medium may contain one or more reducing agents selected from the group consisting of glutathione, cysteine, cystamine, thioglycollate, dithioerythritol and dithiothreitol.

The prokaryotic host cells are cultured at suitable temperatures. For *E. coli* growth, for example, the preferred temperature ranges from about 20°C to about 39°C, more preferably from about 25°C to about 37°C, even more preferably at about 30°C. The pH of the medium may be any pH ranging from about 5 to about 9, depending mainly on the host organism. For *E. coli*, the pH is preferably from about 6.8 to about 7.4, and more preferably about 7.0.

If an inducible promoter is used in the expression vector of the invention, protein expression is induced under conditions suitable for the activation of the promoter. In one aspect of the invention, the transcription of the light and heavy chain are each under the control of a Pho promoter. Accordingly, the transformed host cells are cultured in a phosphate-limiting medium for induction. Preferably, the phosphate-limiting medium is the C.R.A.P medium, as described in detail below in Examples 1 and 2. A variety of other inducers may be used, according to the vector construct employed, as is known in the art.

Eukaryotic host cells are cultured under conditions suitable for expression of the antibody products of the invention. The host cells used to produce the antibody or antigen binding fragments of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in one or more of Ham et al., 1979, *Meth. Enz.* 58:44, Barnes et al., 1980, *Anal. Biochem.* 102: 255, USPN 4,767,704, USPN 4,657,866, USPN 4,927,762, USPN 4,560,655, or USPN 5,122,469, WO 90/103430, WO 87/00195, and USPN Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as

sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPESTM), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCINTM), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Other supplements may
5 also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The expressed light and heavy chain polypeptides of the present invention are
10 generally but not necessarily secreted into and recovered from the periplasm of the prokaryotic host cells or from the medium of eukaryotic cells. Protein recovery typically involves disrupting the microorganism, generally by such means as osmotic shock, sonication or lysis. Once cells are disrupted, cell debris or whole cells may be removed by centrifugation or filtration. The proteins may be further purified, for example, by affinity
15 resin chromatography. Alternatively, proteins can be transported into the culture media and isolated therein. Cells may be removed from the culture and the culture supernatant being filtered and concentrated for further purification of the proteins produced. The expressed polypeptides can be further isolated and identified using commonly known methods such as polyacrylamide gel electrophoresis (PAGE) and immunoblot assay.

20 In one aspect of the invention, the antibody production is conducted in large quantity by a fermentation process. Various large-scale fed-batch fermentation procedures are available for production of recombinant proteins. Large-scale fermentations have at least 10 liters of capacity, preferably about 100 to 100,000 liters of capacity. These fermentors use agitator impellers to distribute oxygen and nutrients, especially glucose (the
25 preferred carbon/energy source). Small-scale fermentation refers generally to fermentation in a fermentor that is no more than approximately 9 liters in volumetric capacity, and can range from about 1 liter to about 9 liters.

In a fermentation process, induction of protein expression is typically initiated after the cells have been grown under suitable conditions to a desired density, e.g., an OD₅₅₀ of
30 about 180-220, at which stage the cells are in the early stationary phase. A variety of inducers may be used, according to the vector construct employed, as is known in the art and described above. Cells may be grown for shorter periods prior to induction. Cells are usually induced for about 12-50 hours, although longer or shorter induction time maybe used.

35 To improve the production yield and quality of the polypeptides of the invention, various fermentation conditions can be modified. For example, to improve the proper assembly and folding of the secreted antibody polypeptides, additional vectors

overexpressing chaperone proteins, such as Dsb proteins (DsbA, DsbB, DsbC, DsbD and/or OsbG) or FkpA (a peptidylprolyl cis,trans-isomerase with chaperone activity) can be used to co-transform the host prokaryotic cells. The chaperone proteins have been demonstrated to facilitate the proper folding and solubility of heterologous proteins produced in bacterial host cells. Chen et al. (1999) *J. Biol. Chem.* 274:19601-19605; Georgiou et al., U.S. Patent No. 6,083,715; Georgiou et al., U.S. Patent No. 6,027,888; Botbmano and Pluckthun (2000) *J. Biol. Chem.* 275:17100-17105; Ramm and Pluckthun (2000) *J. Biol. Chem.* 275:17106-17113; Arie et al. (2001) *Mol. Microbiol.* 39:199-210.

To minimize proteolysis of expressed heterologous proteins (especially those that are proteolytically sensitive), certain prokaryotic host strains deficient for proteolytic enzymes can be used for the present invention. For example, host cell strains may be modified to effect genetic mutation(s) in the genes encoding known bacterial proteases such as Protease III, OmpT, DegP, Tsp, Protease I, Protease Mi, Protease V, Protease VI and combinations thereof. Some *E. coli* protease-deficient strains are available and described in, for example, Joly et al. (1998), supra; Georgiou et al., U.S. Patent No. 5,264,365; Georgiou et al., U.S. Patent No. 5,508,192; Hara et al., *Microbiol Drug Resistance*, 2:63-72 (1996).

In one embodiment, *E. coli* strains deficient for proteolytic enzymes are used as host cells in the expression system of the invention. Some of these strains are further described in the Examples section below.

The methods of the invention also include methods expressing polynucleotides encoding the variable domain produced or modified in accord with the methods of the invention. In one embodiment, the method comprises expressing a polynucleotide encoding a variable domain with at least one modified FR in a host cell. In another embodiment, the method comprises expressing a modified variable domain, wherein the modified variable domain has a substitution of at least one amino acid proximal to a cysteine residue that participates in an intrachain variable domain disulfide bond. The polynucleotides in the methods can further comprise an expression vector encoding both the modified variable domain and constant domains to express a full length heavy and/or light chain. Host cells can coexpress a polynucleotide encoding a light chain and polynucleotide encoding a heavy chain, one or both formed in accord with the methods of the invention, so that full length completely assembled antibodies are produced by the host cell.

Antibody Purification

In one embodiment, the antibody or antigen binding fragment produced herein is further purified to obtain preparations that are substantially homogeneous for further assays and uses. Standard protein purification methods known in the art can be employed. The following procedures are exemplary of suitable purification procedures: fractionation on

immunoaffinity or ion-exchange columns, ethanol precipitation, reverse phase HPLC, chromatography on silica or on a cation-exchange resin such as DEAE, chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, and gel filtration using, for example, Sephadex G-75.

5 In one aspect, Protein A immobilized on a solid phase is used for immunoaffinity purification of the full-length antibody products of the invention. Protein A is a 41kD cell wall protein from *Staphylococcus aureus* which binds with a high affinity to the Fc region of antibodies. Lindmark et al., *J. Immunol. Meth.* 62:1-13 (1983). The solid phase to which Protein A is immobilized is preferably a column comprising a glass or silica surface, more
10 preferably a controlled pore glass column or a silicic acid column. In some applications, the column has been coated with a reagent, such as glycerol, in an attempt to prevent nonspecific adherence of contaminants.

As the first step of purification, the preparation derived from the cell culture as described above is applied onto the Protein A immobilized solid phase to allow specific
15 binding of the full-length antibody to Protein A. The solid phase is then washed to remove contaminants non-specifically bound to the solid phase. Finally the full-length antibody is recovered from the solid phase by elution.

Activity Assays

20 Antibody and/or antigen binding fragments modified according to methods of the present invention can be characterized for its physical/chemical properties and biological functions by various assays known in the art. Methods for protein quantification are well known in the art. For example, samples of the expressed proteins can be compared for their quantitative intensities on a Coomassie-stained SDS-PAGE. Alternatively, the specific
25 band(s) of interest (e.g., the full-length band) can be detected by, for example, immunoblot gel analysis and quantitative intensities detected by scanning densitometer.

Isolated antibody or antigen binding fragments can be further characterized by a series of assays including, but not limited to, N-terminal sequencing, amino acid analysis, non-denaturing size exclusion high pressure liquid chromatography (HPLC), mass
30 spectrometry, ion exchange chromatography and papain digestion.

In certain embodiments of the invention, the antibody or antigen binding fragment produced herein is analyzed for its biological activity. Preferably, the antibody of the present invention is tested for its antigen binding activity. The antigen binding assays that are known in the art and can be used herein include without limitation any direct or
35 competitive binding assays using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, fluorescent immunoassays, and protein A immunoassays. An

exemplary antigen binding assay is provided below in the Examples section. In some embodiments, the antibody or antigen binding fragment thereof modified according to the methods of the invention has an affinity of the antibody not significantly different from or even better than the parent antibody from which it is derived. If improvement in antigen
5 binding affinity of antibodies or antigen binding fragments with modified framework regions is necessary, it can be achieved by altering residues as needed (discussed *supra*).

In one embodiment, the present invention contemplates a full-length antibody that is aglycosylated. The unique features of the antibody (i.e., having an intact Fc region, yet lacking effector functions) make it a desired candidate for many applications in which the
10 half life of the antibody *in vivo* is important yet the effector functions (i.e., complement and ADCC) are unnecessary or deleterious. In certain embodiments, the Fc activities of the produced full-length antibody are measured to ensure that only the desirable properties are maintained.

15 **Pharmaceutical Formulations**

Therapeutic formulations of the antibody or antigen binding fragment modified according to the methods of the invention are prepared for storage by mixing the antibody having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed.
20 (1980)), in the form of aqueous solutions, lyophilized or other dried formulations. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, histidine and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium
25 chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immnoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine;
30 monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.*, Zn-protein complexes); and/or non-ionic surfactants such as TWEENTM, PLURONICSTM or polyethylene glycol (PEG).

35 The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary

activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by lutes-facial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacrylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the full-length antibody, which matrices are in the form of shaped articles, e.g., films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Uses

The methods of the invention are useful to produce humanized antibodies and to improve the yield of antibodies or antigen binding fragments when produced in cell culture. In particular, many antibodies useful therapeutically are produced on a large scale. The methods of the inventions are useful to improve the yield of antibodies produced in both small and large-scale cell culture. The methods can be applied to antibodies or antigen

binding fragments produced in prokaryotic or eukaryotic cells. The antibody products produced by the methods of the inventions have many uses some of which are described below.

5 An antibody or antigen binding fragment modified in accord with the present invention may be used, for example, to purify, detect, and target a specific polypeptide it recognizes, including both *in vitro* and *in vivo* diagnostic and therapeutic methods.

In one aspect, an antibody or antigen binding fragment of the invention can be used in immunoassays for qualitatively and quantitatively measuring specific antigens in biological samples. Conventional methods for detecting antigen-antibody binding includes, 10 for example, an enzyme linked immunosorbent assay (ELISA), an radioimmunoassay (RIA) or tissue immunohistochemistry. Many methods may use a label bound to the antibody for detection purposes. The label used with the antibody is any detectable functionality that does not interfere with its binding to antibody. Numerous labels are known, including the radioisotopes ^{32}P , ^{32}S , ^{14}C , ^{125}I , ^3H and ^{131}I , fluorophores such as rare earth chelates or 15 fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luciferases, e.g., firefly luciferase and bacterial luciferase (U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, .beta.-galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic 20 oxidases such as uricase and xanthine oxidase, lactoperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, imaging radionuclides (such as Technecium) and the like.

Conventional methods are available to bind these labels covalently to the antibody polypeptides. For instance, coupling agents such as dialdehydes, carbodilimides, 25 dimaleimides, bis-imidates, bis-diazotized beozidine, and the like may be used to tag the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels. See, for example, U.S. Pat. No. 3,940,475 (fluorimetry) and U.S. Pat. No. 3,645,090 (enzymes); Hunter et al. *Nature* 144: 945 (1962); David et al. *Biochemistry* 13:1014-1021 (1974); Pain et al. *J. Immunol. Methods* 40:219-230 (1981); and Nygren *Histochem. and* 30 *Cytochem* 30:407-412 (1982). Preferred labels herein are enzymes such as horseradish peroxidase and alkaline phosphatase. The conjugation of such label, including the enzymes, to the antibody polypeptide is a standard manipulative procedure for one of ordinary skill in immunoassay techniques. See, for example, O'Sullivan et al., "Methods for the Preparation of Enzyme-antibody Conjugates for Use in Enzyme Immunoassay," in *Methods in* 35 *Enzymology*, ed. J. J. Langone and H. Van Vunakis, Vol. 73 (Academic Press, New York, N.Y., 1981), pp. 147-166. Such bonding methods are suitable for use with the antibody polypeptides of this invention.

Alternative to labeling the antibody, antigen can be assayed in biological fluids by a competition immunoassay utilizing a competing antigen standard labeled with a detectable substance and an unlabeled antibody. In this assay, the biological sample, the labeled antigen standards and the antibody are combined and the amount of labeled antigen standard bound to the unlabeled antibody is determined. The amount of tested antigen in the biological sample is inversely proportional to the amount of labeled antigen standard bound to the antibody.

In one aspect, a full-length antibody of the invention is particularly useful to detect and profile expressions of specific surface antigens *in vitro* or *in vivo*. The surface antigen can be specific to a particular cell or tissue type, therefore serving as a marker of the cell or tissue type. Preferably, the surface antigen marker is differentially expressed at various differentiation stages of particular cell or tissue types. The full-length antibody directed against such surface antigen can thus be used for the screening of cell or tissue populations expressing the marker. For example, the antibody of the invention can be used for the screening and isolation of stem cells such as embryonic stem cells, hematopoietic stem cells and mesenchymal stem cells. The antibody of the invention can also be used to detect tumor cells expressing tumor-associated surface antigens such as HER2, HER3 or HER4 receptors.

In one aspect, an antibody of the invention can be used in a method for inhibiting an antigen in a subject suffering from a disorder in which the antigen activity is detrimental, comprising administering to the subject an antibody of the invention such that the antigen activity in the subject is inhibited. Preferably the antigen is a human protein molecule and the subject is a human subject. Alternatively, the subject can be a mammal expressing the antigen with which an antibody of the invention binds. Still further the subject can be a mammal into which the antigen has been introduced (e.g., by administration of the antigen or by expression of an antigen transgene). An antibody of the invention can be administered to a human subject for therapeutic purposes. Moreover, an antibody of the invention can be administered to a non-human mammal expressing an antigen with which the antibody cross-reacts (e.g., a primate, pig or mouse) for veterinary purposes or as an animal model of human disease. Regarding the latter, such animal models may be useful for evaluating the therapeutic efficacy of antibodies of the invention (e.g., testing of dosages and time courses of administration). Blocking antibodies of the invention that are therapeutically useful include, for example but not limited to, anti-VEGF, anti-IgE, anti-CD 11 and anti-tissue factor antibodies. The antibodies of the invention can be used to diagnose, treat, inhibit or prevent diseases, disorders or conditions associated with abnormal expression and or activity of one or more antigen molecules, including but not limited to malignant and benign tumors; non-leukemias and lymphoid malignancies; neuronal, gust, astrocytal, hypothalamic

and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic and immunologic disorders.

In certain embodiments, an immunoconjugate comprising the antibody conjugated with a cytotoxic agent is administered to the patient. Preferably, the immunoconjugate and/or antigen to which it is bound is/are internalized by the cell, resulting in increased therapeutic efficacy of the immunoconjugate in killing the target cell to which it binds. In a preferred embodiment, the cytotoxic agent targets or interferes with nucleic acid in the target cell. Examples of such cytotoxic agents include any of the chemotherapeutic agents noted herein (such as a maytansinoid or a calicheainicin), a radioactive isotope, or a ribonuclease or a DNA endonuclease.

Antibodies of the present invention can be used either alone or in combination with other compositions in a therapy. For instance, the antibody may be co-administered with another antibody, chemotherapeutic agent(s) (including cocktails of chemotherapeutic agents), other cytotoxic agent(s), anti-angiogenic agent(s), cytokines, and/or growth inhibitory agent(s). Where the full-length antibody inhibits tumor growth, it may be particularly desirable to combine the full-length antibody with one or more other therapeutic agent(s) which also inhibits tumor growth. For instance, anti-VEGF antibodies blocking VEGF activities may be combined with anti-ErbB antibodies (e.g. HERCEPTIN® anti-HER2 antibody) in a treatment of metastatic breast cancer. Alternatively, or additionally, the patient may receive combined radiation therapy (e.g. external beam irradiation or therapy with a radioactive labeled agent, such as an antibody). Such combined therapies noted above include combined administration (where the two or more agents are included in the same or separate formulations), and separate administration, in which case, administration of the full-length antibody can occur prior to, and/or following, administration of the adjunct therapy or therapies.

The antibody or antigen binding fragment (and adjunct therapeutic agent) is/are administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In addition, the full-length antibody is suitably administered by pulse infusion, particularly with declining doses of the antibody. Preferably the dosing is given by injections, most preferably intravenous or subcutaneous injections.

The antibody or antigen binding fragment composition of the invention will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of

the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The antibody need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of antibody present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

For the prevention or treatment of disease, the appropriate dosage of the antibody (when used alone or in combination with other agents such as chemotherapeutic agents) will depend on the type of disease to be treated, the type of antibody, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 µg/kg to 15mg/kg (e.g. 0.1mg/kg-10mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. The preferred dosage of the antibody will be in the range from about 0.05mg/kg to about 10mg/kg. Thus, one or more doses of about 0.5mg/kg, 2.0mg/kg, 4.0mg/kg or 10mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e.g. every week or every three weeks (e.g. such that the patient receives from about two to about twenty, e.g. about six doses of the antibody). An initial higher loading dose, followed by one or more lower doses maybe administered. An exemplary dosing regimen comprises administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the antibody. However, other dosage regimens maybe useful. The progress of this therapy is easily monitored by conventional techniques and assays.

The following examples are intended merely to illustrate the practice of the present invention and are not provided by way of limitation.

EXAMPLES

Example 1

Heavy Chain Aggregation During Expression and Folding of Antibodies

5 Antibodies produced in cell culture can accumulate intracellularly, in the periplasm or in the extracellular medium. Antibody production typically involves expression of the light and heavy chains in the cytoplasm, secretion into the periplasmic space, folding of the
10 light and heavy chains, and assembly of the folded light and heavy chains to form an antibody molecule. Multiple covalent and non-covalent interactions occur between and within the heavy and light chains during these folding and assembly processes. Antibody yield can be greatly affected by the efficiency and fidelity of these processes. Following synthesis of the heavy and light chains, protein aggregation or proteolysis can occur thereby
15 reducing the yield of the antibody. Solubility experiments were carried out in order to assess the efficiency of steps in the antibody production pathway in bacterial cells for the purpose of improving antibody yield.

Materials and Methods

20 A. Expression and solubilization of Anti-TF and anti-VEGF antibodies.

In order to evaluate several parameters of antibody production, including expression, folding, assembly, and yield of antibody molecules, expression vectors encoding anti-Tissue Factor (anti-TF) monoclonal antibody and anti-VEGF monoclonal antibody, VNERK, were transformed into bacterial cells. Since conditions under which a protein is
25 soluble may provide insight into the basis of folding problems, cell cultures of transformants were subject to various procedures to examine the solubility of heavy and light chains.

1. Transformation and Expression

Expression constructs were transformed into strain 33D3 (W3110 kan^R ΔfhuA
30 (ΔtonA) ptr3 lacIq lacL8 ompTΔ (nmpc-fepE) deg P). Transformants were inoculated into 5 ml Luria-Bertani medium plus carbenicillin (50 ug/ml) and grown overnight at 30°C. Each culture was then diluted (1:100) into C.R.A.P. phosphate-limiting media (3.57g (NH₄)₂SO₄, 0.71g NaCitrate-2H₂O, 1.07g KCl, 5.36g Yeast Extract (certified), 5.36g HycaseSF-Sheffield, pH adjusted with KOH to 7.3, qs to 872 ml with SQ H₂O and
35 autoclaved; cooled to 55°C and supplemented with 110 ml 1M MOPS pH 7.3, 11 ml 50% glucose, 7 ml 1 M MgSO₄) plus carbenicillin (50ug/ml) and grown for about 24 hours at 30°C on a culture wheel.

2. Solubilization

For each culture, two 1 O.D.₆₀₀ pellets were centrifuged in a microfuge for 5 minutes. The cell pellets were then placed at -20°C until preparation. Upon removal from -20°C, one pellet from each culture was used for the solubilization study and the other pellet was used for the preparation of whole cell lysate. See Example 2, Section B.

The solubilization experiment consisted of a multi-step process involving lysis of the cells and preparation of each cell fraction. Cell lysis was the first step in the process. The 1 O.D. pellets were resuspended in 225 ul of 50 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 8) + 1 mg/ml lysozyme + 25 ul of 100 mM IAA (Iodoacetic Acid, Sigma I-2512; to prevent disulfide shuffling). The cells were then lysed by sonicating for 2 x 2 minutes at 50% pulse (Sonics & Materials, Inc., Danbury, CT). The samples were kept in an ice water bath during sonication to dissipate the heat generated during the process. Following sonication, the samples were centrifuged in a microfuge for 5 minutes. At this point, the supernatant and pellet constitute the soluble and insoluble fractions, respectively.

3. Preparation of the Soluble Fraction

For gel analysis, about 500 ul of acetone was added to 125 ul of each soluble fraction (about half of the volume generated) to precipitate the protein. The samples were left at room temperature for about 15 minutes followed by centrifugation for 5 minutes in a microfuge. The protein precipitates were then each resuspended in 25 ul of dH₂O + 25 ul of 2X Sample Buffer. The samples were then heated for about 3-5 minutes at about 90°C, vortexed well and allowed to cool to room temperature. A final 5 minute centrifugation was then done and the supernatants were transferred to clean tubes.

4. Preparation of the Insoluble Fraction

The cell pellets, or insoluble fractions, generated following sonication of the cells, were each resuspended in 100 ul of TE (10 mM Tris pH 7.6, 1 mM EDTA) + 20 ul 10% SDS and vortexed well. The samples were then heated at 90°C for about 3 minutes and vortexed hard again. After cooling to room temperature, about 500 ul of acetone was added to the samples to precipitate the protein and they were left at room temperature for about 15 minutes followed by centrifugation in a microfuge for 5 minutes. The pellets were then resuspended in 50 ul of dH₂O + 50 ul of 2X sample buffer. The samples were then heated for about 3-5 minutes at about 90°C, vortexed well and allowed to cool to room temperature. A final 5 minute centrifugation was then done and the supernatants, designated SDS Soluble, were transferred to clean tubes.

The pellets from the final centrifugation of the SDS Soluble Fraction were further processed. These pellets were each resuspended in 40 ul dH₂O + 10 ul 1M DTT + 50 ul 2X

sample buffer. The samples were then heated for about 3-5 minutes at about 90°C, vortexed well, allowed to cool to room temperature and centrifuged for 5 minutes in a microfuge. The supernatants, designated SDS/DTT soluble, were transferred to clean tubes.

5 **B. Immunoblot Analysis**

Following preparation, 5 ul of each sample (soluble, SDS soluble and SDS/DTT soluble) was mixed with 1 ul of 1M DTT and loaded onto a 10 well, 1.0mm NOVEX manufactured 12% Tris-Glycine SDS-PAGE and electrophoresed at about 120 volts for 1.5 - 2 hours. The resulting gels were then used for immunoblots.

10 The SDS-PAGE gels were electroblotted onto a nitrocellulose membrane (NOVEX). The membrane was then blocked using a solution of 1X NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, 0.05% Triton X-100) + 0.5% gelatin for approximately 30 min. - 1 hour rocking at room temperature. Following the blocking step, the membrane was placed in a solution of 1X NET + 0.5% gelatin + anti-Fab antibody (peroxidase-conjugated
15 goat IgG fraction to human IgG Fab; CAPPEL #55223). The anti-Fab antibody dilution ranged from 1:50,000 to 1:1,000,000 depending on the lot of antibody. The membrane was left in the antibody solution overnight at room temperature with rocking. The next morning, the membrane was washed a minimum of 3 x 10 minutes in 1X NET + 0.5% gelatin and then 1 x 15 minutes in TBS (20 mM Tris pH 7.5, 500 mM NaCl). The protein bands bound
20 by the anti-Fab antibody were visualized by using Amersham Pharmacia Biotech ECL detection and exposing the membrane to X-Ray film.

C. Results of immunoblot analysis

Figure 1A shows the results of immunoblot analysis of different fractions from cells
25 expressing the anti-TF antibody. Figure 1B shows the results of immunoblot analysis of different fractions from cells expressing the anti-VEGF (VNERK) antibody. The location of heavy chain and light chains are designated with arrows. In both Figures 1A and 1B, lane 1 is whole cell lysate, lane 2 is the sonication-soluble fraction, lane 3 is the SDS-soluble fraction, and lane 4 is the SDS/DTT soluble fraction.

30 The whole cell lysates from cells expressing anti-TF and the anti-VEGF (VNERK) antibody, shown in lane 1 of both Figures 1A and 1B, serve as a reference for the approximate amount of total heavy and light chain to follow through the experiment. As seen in lane 2 in both Figures 1A and 1B, the results show that significantly more light chain was present in the sonication-soluble fraction than heavy chain. This result suggests that a
35 large percentage of the expressed light chain is correctly folded. On the other hand, heavy chain is observed primarily in the SDS soluble and SDS/DTT soluble fractions. The protein in the SDS soluble fraction implies aggregation through hydrophobic interactions, while

protein found in the SDS/DTT soluble fraction implies that mispaired disulfide bonds either contribute to, or are largely responsible for, the aggregation process. A significant amount of heavy chain was observed in the fraction that was SDS/DTT soluble. This result indicates that following translation, much of the heavy chain forms protein aggregates that are
5 primarily soluble in denaturing and reducing conditions. Following heavy chain synthesis, a significant amount of the heavy chain misfolds and aggregates, leaving free intracellular light chain and thereby reducing antibody yield.

Example 2

10 Preparation of anti-VEGF Antibodies With Improved Yield

The yield of antibodies or antigen binding fragments from cells can be influenced by the *in vivo* folding and/or assembly of the antibody. In order to increase antibody or antigen binding fragment yield, the sequence of the antibody or fragment was modified and
15 the effect on folding and yield on the antibody was examined. As described in Example 1, aggregation of heavy chains may contribute to a decrease in antibody folding, assembly and yield. Targeted modification of the heavy chain variable region FR1 of anti-VEGF antibodies was performed. Antibody variants with different human variable domain subgroup consensus sequences in the FR1 were prepared and the yield of completely
20 assembled antibody products was examined.

MATERIALS AND METHODS

A. Preparation of expression vectors encoding modified anti-VEGF antibodies

To evaluate the effect of different heavy chain FR1 human subgroup consensus
25 sequences on the expression, folding, assembly, and yield of anti-VEGF antibodies, vectors encoding the light and heavy chains for two different anti-VEGF antibodies, VNERK and Y0317, were constructed. Both of these antibodies were originally constructed with framework regions in the heavy chain from human variable domain subgroup III consensus sequence. The FR1 subgroup III residues of the antibodies were substituted with either the
30 FR1 subgroup I or subgroup II residues at those positions where the sequence differed.

The human variable domain subgroup sequences can be found in the Kabat database available at a number of locations on the internet, such as <http://www.kabatdatabase.com>, and have been described in Kabat et al., Sequences of proteins of immunological interest, Ed.5. Public Health Service, National Institutes of Health, Bethesda, MD, 1991. A
35 consensus variable domain sequence for each subgroup was constructed by selecting the most frequently occurring amino acid for each position in the variable domain. The FR1 sequences corresponding to amino acids 1-25 for each subgroup are:

Subgroup I QVQLVQSGAEVKKPGASVKVSCAS (SEQ ID NO: 1)

Subgroup II QVQLQESGPGLVKPSQTLSTCTVS (SEQ ID NO: 2)

Subgroup III EVQLVESGGGLVQPGGSLRLSCAAS (SEQ ID NO: 3)

The anti-VEGF antibody VNERK is a higher affinity variant of the humanized anti-VEGF antibody described in Presta et al, *Cancer Res.*, 57, 4593 (1997).

Anti-VEGF antibody Y0317 is described in pending U.S. Patent Application Publication No. US2002/0032315, Application. Ser. No. 09/056,160 filed April 6, 1998 and Chen et al, (1999) *J. Mol. Biol.*, 293:865-881. Briefly, Y0317 is a humanized antibody isolated using phage display methods starting with a template antibody with HVRs from murine anti-VEGF monoclonal antibody A.4.6.1; human consensus kappa subgroup I light chain framework and constant sequences; and human consensus subgroup III heavy chain framework and constant sequences. The antibody sequence also has substitutions at H101Y and S105T.

The construction of a separate cistron vector is illustrated in Figure 2 and has been described in Simmons et al., *J. Immunol. Methods* 263:133-147 (2002) and in WO 02/061090 published August 8, 2002. Vectors with separate cistrons were designed to provide independent transcription and translation of the light and heavy chain genes of an antibody. For all vector constructions, expression cassettes encoding anti-VEGF antibody VNERK and Y0317 heavy and light chain sequences were cloned into the *E. coli* plasmid pBR322 at the EcoRI site. Sutcliffe, *Cold Spring Harbor Symp. Quant. Biol.* 43:77-90 (1978).

The expression cassette in each designed vector contains at least the following basic components: (1) *phoA* promoter for the control of transcription; (2) λt_0 terminator to end transcription; (3) the Shine-Dalgarno sequence from the *E. coli trp* or the heat stable enterotoxin II (STII) gene, or a combination of both, to facilitate translation. The basic components of bacterial expression cassettes are known in the art and have been described in, for example, Kikuchi et al., *Nucleic Acids Res.* 9(21):5671-5678 (1981) (for *phoA* promoter); Scholtissek and Grosse, *Nucleic Acids Res.* 15:3185 (1987) (for λt_0 terminator); Yanofsky et al., *Nucleic Acids Res.* 9:6647-6668 (1981) (for *trp*); Picken et al., *Infect. Immun.* 42:269-275 (1983) for STII; and Chang et al., *Gene* 55:189-196 (1987) (for combination use of *trp* and STII Shine-Dalgarno sequence). Additionally, the STII signal sequence or silent codon variants thereof precedes the coding sequence for both light and

heavy chains in all constructs described and directs the secretion of the protein into the periplasm. Picken et al., *Infect. Immun.* 42:269-275 (1983); Simmons and Yansura, *Nature Biotechnology* 14:629-634 (1996). In this design, the cistron unit for each chain is under the control of its own PhoA promoter and is followed by a λt_0 terminator. Construction of

5 suitable vectors containing one or more of the above listed components employs standard ligation techniques and or other molecular cloning techniques known in the art. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

Separate cistron vectors encoding the heavy and light chains from anti-VEGF

10 antibodies VNERK and Y0317 were constructed as described in U.S. Patent No. 5,747,662.

The vector designated pxVG11VNERK is a separate cistron vector encoding the VNERK heavy and light chain sequences. The nucleotide (SEQ ID NO: 4) and amino acid sequences (SEQ ID NO: 5) for the heavy and light chain of VNERK are shown in Figure 15. The vector designated pxVG2AP11 is a separate cistron vector encoding the heavy and light

15 chain sequences from Y0317. The nucleotide (SEQ ID NO: 6) and amino acid sequence (SEQ ID NO: 7) for the heavy and light chains of Y0317 are shown in Figure 16.

Additional expression vectors were constructed to determine the effects of replacement of heavy chain consensus FR1 subgroup III amino acid residues with the heavy chain consensus subgroup I or subgroup II amino acid residues.

20 In order to change the heavy chain FR1 subgroup III sequence to that of the subgroup I sequence, 12 amino acid substitutions in FR1 sequence were required in the anti-VEGF VNERK heavy chain variable domain. Replacement of the heavy chain FR1 subgroup III consensus sequence in pxVG11VNERK with heavy chain FR1 subgroup I consensus sequence QVQLVQSGAEVKKPGASVKVSCKAS (SEQ ID NO: 1) was

25 performed in a single step by replacing nucleotide sequence encoding the heavy chain FR1 subgroup III sequence with a double stranded oligonucleotide encoding the FR1 subgroup I sequence. The resulting plasmid, pVKFR1-2, encodes heavy and light chain variable domain amino acid sequences (SEQ ID NO: 9) shown in Figure 17. The nucleotide sequence for pVKFR1-2 (SEQ ID NO: 8) is also shown in Figure 17.

30 In order to change the heavy chain FR1 subgroup III sequence to that of the subgroup II sequence, 11 amino acid substitutions in FR1 sequence are required. Replacement of the heavy chain FR1 subgroup III in pxVG11VNERK with heavy chain FR1 subgroup II consensus sequence QVQLQESGPGLVKPSQTLSTCTVS (SEQ ID NO: 2) was performed in a similar manner. The resulting plasmid pVKSGII, encodes heavy and

35 light chain variable domain amino acid sequences (SEQ ID NO: 11) shown in Figure 18. The nucleotide sequence encoding the heavy and light chains (SEQ ID NO: 10) is also shown in Figure 18.

Replacement of the heavy chain FR1 subgroup III in pxVG2AP11 (Y0317 anti-VEGF antibody) with heavy chain FR1 subgroup I sequence QVQLVQSGAEVKKPGASVKVSKAS (SEQ ID NO: 1) was performed in a similar manner. The resulting plasmid, pY0FR1-2 includes the polynucleotide sequence (SEQ ID NO: 12), encoding heavy and light chain variable domain amino acid sequences (SEQ ID NO: 13) shown in Figure 19.

B. Expression, Folding and Yield of Humanized anti-VEGF Antibody Variants

Expression vectors as described in Section A were transformed into bacteria to determine the expression, folding, assembly and yield of the anti-VEGF antibodies having the indicated changes in the heavy chain FR1 sequence.

1. Shake Flask Inductions

Plasmids prepared as described in Section A were transformed into strain 33D3 (W3110 kan^R ΔfhuA (ΔtonA) ptr3 lacIq lacL8 ompTΔ (nmpc-fepE) deg P). Transformants were inoculated into 5 ml Luria-Bertani medium plus carbenicillin (50 ug/ml) and grown overnight at 30° C. Each culture was then diluted (1:100) into C.R.A.P. phosphate-limiting media (3.57g (NH₄)₂SO₄, 0.71g NaCitrate-2H₂O, 1.07g KCl, 5.36g Yeast Extract (certified), 5.36g HycaseSF-Sheffield, pH adjusted with KOH to 7.3, qs to 872 ml with SQ H₂O and autoclaved; cooled to 55°C and supplemented with 110 ml 1M MOPS pH 7.3, 11 ml 50% glucose, 7 ml 1 M MgSO₄) plus carbenicillin (50ug/ml) and grown for about 24 hours at 30°C on a culture wheel.

2. Preparation of Samples for SDS-PAGE

Non-reduced whole cell lysates from induced cultures were prepared as follows: (1) 1 OD₆₀₀ -ml pellets were centrifuged in a microfuge tube; (2) each pellet was resuspended in 90 ul TE (10mM Tris pH 7.6, 1mM EDTA); (3) 10 ul of 100 mM iodoacetic acid (Sigma I-2512) was added to each sample to block any free cysteines and prevent disulfide shuffling; (4) 20 ul of 10% SDS was added to each sample. The samples were vortexed, heated to about 90°C for about 3 minutes and then vortexed again. After the samples had cooled to room temperature, about 750-1000 ul acetone was added to precipitate the protein. The samples were vortexed and left at room temperature for about 15 minutes. Following centrifugation for 5 minutes in a microcentrifuge, the supernatant of each sample was aspirated off and each protein pellet was resuspended in 50 ul dH₂O + 50 ul 2X NOVEX sample buffer. The samples were then heated for about 3-5 minutes at about 90°C, vortexed

well and allowed to cool to room temperature. A final 5 minute centrifugation was then done and the supernatants were transferred to clean tubes.

Reduced samples were prepared by following steps similar to the steps described above for non-reduced samples, except that 10 ul of 1M DTT was added to the cell resuspension solution in Step (2) and the addition of IAA was omitted in Step (3). Reducing agent was also added to a concentration of 100 mM when the protein precipitate was resuspended in 2X NOVEX sample buffer + dH₂O.

3. Immunoblot Analysis

Following preparation, 5-10 ul of each sample was loaded onto a 10 well, 1.0 mm NOVEX manufactured 12% Tris-Glycine SDS-PAGE and electrophoresed at about 120 volts for 1.5 - 2 hours. The resulting gels were then used for immunoblots.

The SDS-PAGE gels were electroblotted onto a nitrocellulose membrane (NOVEX). The membrane was then blocked using a solution of 1X NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, 0.05% Triton X-100) + 0.5% gelatin for approximately 30 min. - 1 hour rocking at room temperature. Following the blocking step, the membrane was placed in a solution of 1X NET + 0.5% gelatin + anti-Fab antibody (peroxidase-conjugated goat IgG fraction to human IgG Fab; CAPPEL #55223). The anti-Fab antibody dilution ranged from 1:50,000 to 1:1,000,000 depending on the lot of antibody. The membrane was left in the antibody solution overnight at room temperature with rocking. The next morning, the membrane was washed a minimum of 3 x 10 minutes in 1X NET + 0.5% gelatin and then 1 x 15 minutes in TBS (20 mM Tris pH 7.5, 500 mM NaCl). The protein bands bound by the anti-Fab antibody were visualized by using Amersham Pharmacia Biotech ECL detection and exposing the membrane to X-Ray film.

The relative amounts of the completely assembled product in bands detected on the immunoblot were measured using scanning densitometry. The net intensity (in pixels) of each full-length completely assembled antibody band (i.e. the top band on the immunoblots) was determined using the following tools: Kodak Digital Science Image Station 440CF, Software: Kodak Digital Science 1D Image Analysis Software (v. 3.0.2), System: Microsoft Windows 95. The net intensity of each of the full-length completely assembled antibody bands for the variant antibodies with framework changes were then divided by the net intensity of the unmodified antibody to provide a value for the yield. The unmodified or control antibody was assigned a value of 1.

4. Large-Scale Fermentation Conditions

The organisms used for large scale fermentations included 61D6 W3110 kan^R ΔfhuA (ΔtonA) ptr3 lacIq lacL8 ompT Δ(nmpc-fepE) deg P as described in WO 02/061090.

For each 10-liter fermentation, 0.5 mL of frozen stock culture (containing 10-15% DMSO) was thawed and used to inoculate a 2L shake flask containing 500 ml of LB medium supplemented with either 0.5 ml of tetracycline solution (5 mg/ml) or 10 mL of ampicillin solution (2 mg/mL) and 2.5 ml 1M sodium phosphate solution. This seed culture
5 was grown for approximately 16 hours at 30°C with shaking and was then used to inoculate the 10-liter fermentor.

The fermentor initially contained approximately 7.0 liters of medium containing 1.1 g of glucose, 100 ml of 1M magnesium sulfate, 10 ml of a trace element solution (100 ml hydrochloric acid, 27 g ferric chloride hexahydrate, 8 g zinc sulfate heptahydrate, 7 g cobalt
10 chloride hexahydrate, 7 g sodium molybdate dihydrate, 8 g cupric sulfate pentahydrate, 2 g boric acid, 5 g manganese sulfate monohydrate, in a final volume of 1 liter), either 20 ml of a tetracycline solution (5 mg/ml in ethanol) or 250 mL of an ampicillin solution (2 mg/mL), 1 bag of HCD salts, (37.5 g ammonium sulfate, 19.5 g potassium phosphate dibasic, 9.75 g sodium phosphate monobasic dihydrate, 7.5 g sodium citrate dihydrate, 11.3 g potassium
15 phosphate monobasic), 200 g of NZ Amine A (a protein hydrolysate), and 100 grams of Yeast Extract. Fermentations were performed at 30 °C with 20 slpm of air flow and were controlled at a pH of 7.0 ± 0.2 (although occasional excursions beyond this range occurred in some cases). The back pressure of the fermentor was maintained at 1 bar gauge and the agitation rate was set to 650 rpm. The back pressure of the fermentor and agitation rate can
20 also be varied to manipulate the oxygen transfer rate in the fermentor, and, consequently, control the cellular respiration rate.

Following inoculation of the fermentor with the cell-containing medium from the shake flask, the culture was grown in the fermentor to high cell densities using a computer-based algorithm to feed a concentrated glucose solution to the fermentor. Ammonium
25 hydroxide (58% solution) and sulfuric acid (24% solution) were also fed to the fermentor as needed to control pH. Additions of L-61 (an antifoam – others can be used) were also used in some cases to control foaming. When the culture reached a cell density of approximately 40 OD550, an additional 100 ml of 1M magnesium sulfate was added to the fermentor. Additionally, a concentrated salt feed (12.5 g ammonium sulfate, 32.5 g potassium
30 phosphate dibasic, 16.25 g sodium phosphate monobasic dihydrate, 2.5 g sodium citrate dihydrate, 18.75 g potassium phosphate monobasic, 10 ml of 2.7% ferric chloride and 10 ml of trace elements in a final volume of 1250 ml) was added to the fermentor and started at a rate of 2.5 ml/min when the culture reached approximately 20 OD550 and continued until approximately 1250 ml were added to the fermentation. Fermentations were typically
35 continued for 70-80 hours. During the fermentation, once the dissolved oxygen set point for the fermentation was reached, the concentrated glucose solution was fed based on the dissolved oxygen probe signal in order to control the dissolved oxygen concentration at the

set point. Consequently, in this control scheme, manipulations of fermentor operating parameters such as the agitation rate or back pressure which affect the oxygen transfer capacity in the fermentation correspondingly also manipulated the oxygen uptake rate or metabolic rate of the cells. A mass spectrometer was used to monitor the composition of the off-gas from the fermentations and enable the calculation of the oxygen uptake and carbon dioxide evolution rates in the fermentations.

5. AME5-RP Assay

Samples of the soluble fractions were submitted for analysis by an AME5-RP assay. This assay is a dual column HPLC assay where the first column is an affinity column that captures light chain and the second column is a reversed-phase column. An Integral Workstation was configured in the dual column mode. The solvent reservoirs were: Solvent 1A, affinity loading buffer; Solvent 1B, reversed-phase aqueous buffer and affinity elution buffer, 0.1% TFA in water; Solvent 2A, water; Solvent 2B, reversed-phase organic elution buffer, 0.09% TFA/80% acetonitrile. The first column was the affinity column (30 x 2.1 mm) containing an immobilized anti-light-chain (kappa) Fab antibody (AME5) immobilized on controlled pore glass. All procedures involving the affinity column were performed at ambient temperature. The second column was the reversed-phase column containing the polymer based POROS R220 packing material (30 x 2.1 mm). The reversed-phase column temperature was maintained at 60°C.

The affinity column was equilibrated in 30% loading buffer (5ml) and a 50 ul sample was loaded at a flow rate of 0.1 ml/min. The flow-through was directed to waste. After the sample was loaded the affinity column was washed with 30% loading buffer (2 ml), followed by 100% loading buffer (5 ml) to reduce non-specifically bound components. A final wash with water prepared the affinity column for elution (3 ml). The affinity column was now connected to the reversed-phase column (by valve switching) and eluted with elution buffer (2 ml) at a flow rate of 2 ml/min to transfer the affinity captured components to the reversed phase column. During this transfer step the Integral UV detector is located after the affinity column and before the reversed-phase column and hence monitors the elution of the affinity column (which becomes the load to the reversed-phase column). In addition to this detector, a second detector was added after the reversed-phase column to monitor its flow-through to confirm that all the components eluted from the affinity column were in fact captured by the reversed-phase column.

Re-equilibration of the affinity column was subsequently performed with loading buffer (4 ml) after removing its connection to the reversed-phase column.

The loaded reversed-phase column was washed with aqueous 0.1% TFA (2 ml). The flow rate was set to 1 ml/min and a rapid gradient (1 min) was run to 35% solvent 2B (0.1%

TFA/80% acetonitrile) followed by a shallow gradient to 50% solvent 2B over 14 min. Elution is completed by a gradient to 90% solvent 2B over 4 min. The reversed phase column was then returned to initial conditions over 1 min. and re-equilibrated for 3 min at 2 ml/min. The column eluate was monitored at 280 and 214 nm. Quantitation was performed by comparison of the integrated peak areas with those of standards of known concentrations.

6. Antibody Binding Affinity Assays

BIAcore™ binding analysis

The VEGF-binding affinities of full length antibodies produced in bacterial cells were calculated from association and disassociation rate constants measured using a BIAcore™-2000 surface plasmon resonance system (BIAcore, Inc., Piscataway, NJ) as described in Chen et al, (1999) *J. Mol. Bio.* 293:865-881). A biosensor chip was activated for covalent coupling of VEGF using N-ethyl-N'-(3-dimethylaminopropyl)-carbo-dimide hydrochloride (EDC) and N-hydroxysuccinimid (NHS) according to the supplier's (BIAcore, Inc., Piscataway, NJ) instructions. VEGF (109) or VEGF (165) was buffer-exchanged into 20 mM sodium acetate, pH 4.8 and diluted to approximately 50 µl/minute to achieve approximately 700-1400 response units (RU) of coupled protein. A solution of 1 M ethanolamine was injected as a blocking agent.

For kinetics measurements, twofold serial dilutions of full length antibodies were injected in PBS/Tween buffer (0.05% Tween-20 in phosphate-buffered saline) at 25°C or 37°C at a flow rate of 10 µl/minute. Equilibrium dissociation constants, K_d values from SPR measurements were calculated as k_{off}/k_{on} .

C. Results

Figure 3 shows the results of a representative immunoblot of lysates from induced cells transformed with pxVG11VNERK (SGIII/lane 1), pVKFR1-2 (SGI/lane 2), and pVKSGII(SGII/lane 3); lane 4 is a negative control (cells transformed with the plasmid, pBR322). Samples were prepared under reduced or non-reduced conditions. In Figure 3A, lysates prepared under reduced conditions are shown. Locations of the heavy and light chains are shown. In Figure 3B, nonreduced lysates were separated on a polyacrylamide gel. The drawings to the right of the figure refer to the various antibody structures, including the completely assembled full-length product, various species lacking one or more disulfide bonds including a heavy-light band, monomeric heavy chain, light chain dimer and monomeric light chain, and the relative migration of these structures on the non-reducing gel. The band at the top of the non-reducing gel typically represents the full-length completely disulfide bonded antibody product.

When the cell lysates were prepared under reducing conditions and separated, the results show (Figure 3A) that there were no significant differences in the amounts of heavy or light chains produced in the cells when the three antibodies were compared. When the same cell lysates were prepared under non-reducing conditions and separated, the results show that the unmodified antibody with heavy chain FR1 subgroup III produced a number of products including the full-length completely assembled product. (Figure 3B, lane 1). Replacement of the heavy chain FR1 subgroup III sequence with heavy chain FR subgroup I sequence increased the yield of the assembled antibody products including the completely assembled full-length product. (Figure 3B, lane 2) Replacement of heavy chain FR1 subgroup III sequence with the subgroup II sequence resulted in production of little or no folded and assembled products. (Figure 3B, lane 3).

The yield of anti-VEGF(VNERK) antibody with the FR1 subgroup I sequence was about 2 fold greater than the anti-VEGF(VNERK) antibody with subgroup III FR1 sequences based on densitometer scans of the completely assembled bands as shown in Table 2 below. The value of n is the number of different gels that were scanned.

TABLE 2

	xVEGF (VNERK)	
20	<u>Plasmid</u>	<u>Yield</u>
	pxVG11VNERK (FR1=SGIII)	1
	pVKFR1-2 (FR1=SGI)	2.2 +/- 0.2 (n=9)
	pVKSGII (FR1=SGII)	Band not detected (n=2)
25	xVEGF (Y0317)	
	<u>Plasmid</u>	<u>Yield</u>
	pxVG2AP11 (FR1=SGIII)	1
30	pY0FR1-2 (FR1=SGI)	8.2 +/- 1.9 (n=2)

These results show that replacement of heavy chain FRI subgroup III sequences with heavy chain FR1 subgroup I sequences in anti-VEGF antibody VNERK increases the yield of completely assembled antibody products. The results from the samples separated under reducing conditions indicate approximately equivalent amount of heavy and light chains were produced in these cells. The increase in full-length completely assembled antibody products demonstrated by the non-reduced samples (Figure 3B, lane 2) combined with essentially unchanged overall expression level of heavy and light chains demonstrated by the reduced samples (Figure 3A, lane 2) suggests that the substitution of heavy chain FR1 residues resulted in an increase in folding and/or assembly efficiency which may account for the observed increase in yield.

To determine if the FR1 subgroup substitutions had any effect on the binding affinity for VEGF antigen, an affinity assay was performed using the expressed antibodies obtained from bacterial cells. VNERK antibodies having the original FR1 subgroup III sequence (pxVG11VNERK) and the subgroup I sequence substitution (pVKFR1-2) showed
5 no significant difference in binding affinity for the VEGF antigen. The anti-VEGF antibody with subgroup III had a binding affinity of 0.12 nM and the anti-VEGF with subgroup I had a binding affinity of 0.19 nM (data not shown).

Figure 4 shows the results of immunoblot analysis of lysates from induced cells transformed with pxVG2AP11 (Y0317 antibody) (original FR1-SGIII/lane 1) or pY0FR1-2
10 (new FR1-SGI/lane 2). The reduced samples, shown in Figure 4A, demonstrate that approximately equivalent levels of heavy and light chain are expressed from both constructs. Non-reduced lysates were also separated on a polyacrylamide gel as shown in Figure 4B. The results in Figure 4B demonstrate that replacement of the heavy chain FR1 subgroup III sequence with the FR1 subgroup I sequence increases the yield of the folded
15 Y0317 antibody. As shown in Table 2, the yield of the completely disulfide bonded product was increased about 8 fold over the antibody with the subgroup III sequence. As stated previously for anti-VEGF VNERK, this increased yield is likely due to improved heavy chain folding and /or assembly efficiency.

The results show that replacement of the heavy chain FR1 subgroup III consensus
20 sequence with the subgroup I consensus sequence in two different anti-VEGF antibodies, VNERK and Y0317, improved the yield of assembled antibody. The substitutions did not significantly alter the amount of heavy and light chains expressed and did not significantly change the antigen binding affinity of the anti-VEGF VNERK antibody. Additionally, in contrast to the subgroup I replacement, the replacement of the FR1 subgroup III consensus
25 sequence with the subgroup II consensus sequence significantly reduced the yield of assembled antibody.

Similar results are seen when the antibodies were produced in cells by large scale fermentation methods. Bacterial cells transformed with plasmids encoding anti-VEGF antibody VNERK (pxVG11VNERK; Fermentation run #AB422) and anti-VEGF antibody
30 VNERK antibody modified to have heavy chain FR1 subgroup I consensus sequence (pVKFR1-2; Fermentation runs #AB249 and # AB444) were grown under large scale fermentation conditions. The results are shown in Figure 14. In Figure 14A, the non-reduced soluble fraction samples from bacterial paste were run on a SDS-PAGE and stained with Coomassie Blue. In Figure 14B, the SDS-PAGE immunoblot of the non-reduced
35 lysates stained with detectably labeled anti-Fab antibody is shown. The results show that there was an increase in yield in the anti-VEGF VNERK antibody with heavy chain FR1 subgroup I consensus sequence when produced on a large scale. The soluble fractions were

also submitted for AME5RP assay. No peak was detectable for the soluble fraction sample from cells transformed with plasmid pxVGIIVNERK (AB422). The extracts of cells transformed with pVKFR1-2 yielded about 47 (AB249) or 49 (AB444) mg/L of antibody.

These results confirm the finding seen with smaller scale production runs and show that an increase in antibody yield is seen when the heavy chain FR1 amino acid results are modified from the subgroup III consensus sequence to the subgroup I consensus sequence in anti-VEGF antibodies.

D. Selection of a heavy chain FR1 sequence for anti-VEGF antibody VNERK based on HVR consensus subgroup I-III comparisons

The anti-VEGF antibody VNERK is a humanized antibody with HVR region sequences from a mouse monoclonal antibody and heavy chain framework regions from consensus sequence subgroup III. As discussed above, substitution of the heavy chain FR1 subgroup III sequences with subgroup I sequence surprisingly resulted in a significant increase in assembled antibody yield when the antibodies were produced in *E. coli*.

When the HVR1 sequence of the antibody VNERK was compared to each of the consensus sequence HVR1 regions of the heavy chain subgroups, it was discovered that the HVR1 region of VNERK had the most sequence identity with HVR1 region of the subgroup I heavy chain consensus sequence. VNERK has a heavy chain HVR1 including amino acid residues 26-35 having the following sequence: GYTFTNYGIN. (SEQ ID NO: 14) This sequence was compared to the corresponding residues 26-35 of each of human heavy chain consensus sequences from subgroups I-III. The percentage identity was determined by calculating the number of amino acids that were identical at the same position between the VNERK sequence and each subgroup consensus sequence. The identity is based on the total number of identical amino acids divided by the number of amino acids in the relative HVR1 subgroup consensus sequence. Table 3 shows alignment of these HVR1 sequences and the percentage identity between the pairs.

TABLE 3

Sequence	HVR Alignment	% Identity
Subgroup I HVRH1:	GYTFTSYAIS (SEQ ID NO: 15)	70%
VNERK HVR1:	 GYTFTNYGIN (SEQ ID NO: 14)	7/10
Subgroup II HVRH1:	GGSVSSYWSN (SEQ ID NO: 16)	18%
VNERK HVR1:	 GYTFTNYGIN (SEQ ID NO: 14)	2/11
Subgroup III HVRH1:	GFTFSSYAMS (SEQ ID NO: 17)	40%
VNERK HVR1:	 GYTFTNYGIN (SEQ ID NO: 14)	4/10

The heavy chain HVR1 of anti-VEGF (VNERK) GYTFTNYGIN (SEQ ID NO: 14) shows the most sequence identity with the HVR1 consensus sequence of Subgroup I and the least sequence identity with the HVR1 consensus sequence of Subgroup II. As described above, substitution of the heavy chain FR1 subgroup III of anti-VEGF (VNERK) with FR1 of subgroup I enhanced yield while substitution of FR1 with Subgroup II reduced yield. This result suggests a correlation between the FR1 sequence selected and the yield of assembled antibody and folding efficiency.

As part of the humanization process, several substitutions were made to the consensus subgroup III sequences in FR2 and FR3. The substitutions at these positions were from the corresponding position in the murine antibody. Four of the seven substitutions also happen to involve a change from a SGIII consensus residue to a SGI consensus residue. Table 4 shows the four FRs of VNERK following humanization.

TABLE 4

Sequence	Heavy Chain Alignment
<u>FR1</u> SGIII VNERK	EVQLVESGGGLVQPGGSLRLSCAAS EVQLVESGGGLVQPGGSLRLSCAAS
<u>FR2</u> SGIII VNERK SGI	WVRQAPGKGLEWVS WVRQAPGKGLEWVG G
<u>FR3</u> SGIII VNERK SGI	71 78 RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR RFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAK I A T S A R
<u>FR4</u> SGIII VNERK	WGQGTLLTVSS WGQGTLLTVSS

Some of the changes made during humanization to improve antibody affinity were made at positions that did not differ in amino acid sequence between the subgroup I and subgroup III sequence, such as at position 94 (Kabat numbering). This suggests that some additional modifications at positions other than those that differ between the selected subgroup consensus sequence and the antibody variable domain sequence may be made in order to improve binding affinity in the humanized antibody or antigen binding fragment.

When applying this method to humanized antibodies or antigen binding fragments, some of the FR region substitutions at the positions identified in accord with the methods of the invention may have already been made to improve antigen binding affinity, the improvement of the yield may be less than that would be expected if the changes to the subgroup III sequence had not already been made to the humanized antibody. Designing the

anti-VEGF VNERK antibody with heavy chain FR regions from the human consensus subgroup I instead of subgroup III may have shortened the time to producing a humanized antibody or antigen binding fragment that can be produced in high yield in cell culture.

The HVR1 sequence of the heavy chain variable domain antibody Y0317 was also compared to consensus sequence HVR1 regions of each of the heavy chain subgroups. The HVR1 region of Y0317 had the most sequence identity with HVR1 region of the subgroup I. Y0317 includes a heavy chain HVR1 including amino acid residues 26-35 having the following sequence: GYDFTHYGMN. (SEQ ID NO: 18) This sequence was compared to the corresponding residues 26-35 of each of human heavy chain consensus sequences from subgroups I-III. The percentage identity was determined by calculating the number of amino acids that were identical at the same position between the Y0317 HVR1 sequence and each subgroup consensus sequence. The identity is based on the total number of identical amino acids divided by the number of amino acids in the relative HVR1 subgroup consensus sequence. Table 5 shows alignment of these HVR1 sequences and the percentage identity between the pairs.

TABLE 5

Sequence	HVR Alignment	% Identity
Subgroup I HVRH1:	GYTFTSYAIS (SEQ ID NO: 15)	50%
Y0317 HVR1:	GYDFTHYGMN (SEQ ID NO: 18)	5/10
Subgroup II HVRH1:	GGSVSSYWSN (SEQ ID NO: 16)	18%
Y0317 HVR1:	GYDFTHYGMN (SEQ ID NO: 18)	2/11
Subgroup III HVRH1:	GFTFSSYAMS (SEQ ID NO: 17)	40%
Y0317 HVR1:	GYDFTHYGMN (SEQ ID NO: 18)	4/10

These results suggest that FR1 sequences that provide improved antibody assembly and yield can be identified by comparing the HVR1 region sequence of the antibody with corresponding consensus sequence subgroup sequences and identifying the subgroup with the most sequence identity to the HVR1 sequence of the antibody.

Example 3

Selection of a heavy chain FR1 sequence for antibodies based on comparison of HVR1 antibody sequences to HVR1 consensus subgroup I-III sequences

Substitution of heavy chain FR1 consensus sequence subgroup III with consensus sequence subgroup I in anti-VEGF antibodies increased assembled antibody yield, presumably due to improved folding efficiency. Framework region sequences that provide

for improved yield for other antibodies or antigen binding fragments can be identified based upon identifying the consensus sequence subgroup that has the most sequence identity in the HVR1 region of the variable domain when the HVR1 region of the antibody or fragment is compared to the corresponding HVR1 sequences of each of the subgroup consensus sequences. The method of identifying a heavy chain FR1 sequence for increasing the yield of antibodies based on HVR1 consensus subgroup I-III comparisons was tested for an anti-IgE antibody, E25.

A. Identification of a heavy chain FR1 sequence for humanized anti-IgE antibody E25 based on HVR consensus subgroup I-III comparisons

The anti-IgE antibody E25 is described in Shields R.L., et al., 1995 (Int Arch Allergy Immunol; 107:308) and U. S. Patent No. 6,172,213. The sequence of this antibody is provided in U.S. Patent No. 6,172,213. Antibody E25 is a humanized antibody specific for IgE and was prepared with HVR sequences from mouse anti-human IgE monoclonal antibody MaE11 and framework sequences from human consensus subgroup I for the kappa light chain and human consensus subgroup III sequences for the heavy chain. As part of the humanization process, at least one residue in the FR1 of heavy chain was changed. This modification, A24V, changes a subgroup III residue, Alanine, to the murine residue, Valine, at this position. The murine residue at this position also corresponds to the residue in human variable domain subgroup II consensus sequence.

E25 includes a heavy chain variable region HVR1 including amino acid residues 26 to 35 having the following sequence: GYSITSGYSWN. (SEQ ID NO: 19) This E25 heavy chain HVR1 sequence was compared to the each of the heavy chain HVR1 subgroup consensus sequences I-III as shown below.

The alignment was performed by pairing the E25 HVR1 sequence with the corresponding sequence in each subgroup I-III consensus sequence. The percentage identity was determined by calculating the number of amino acids that were identical at the same position between the E25 sequence and each subgroup consensus sequence. The identity is based on the total number of identical amino acids divided by the number of amino acids in the relative HVR1 subgroup consensus sequence. Table 6 shows alignment of these HVR1 sequences and the percentage identity between the pairs.

TABLE 6

Sequence	HVR Alignment	% Identity
Subgroup I HVRH1:	GYTFTSYAIS (SEQ ID NO: 15)	40%
E25 HVR1:	 GYSITSGYSWN (SEQ ID NO:19)	(4/10)
Subgroup II HVRH1:	GGSVSSYWSWN (SEQ ID NO:16)	55%
E25 HVR1:	 GYSITSGYSWN (SEQ ID NO:19)	6/11
Subgroup III HVRH1:	GFTFSSYAMS (SEQ ID NO: 17)	20%
E25 HVR1:	 GYSITSGYSWN (SEQ ID NO:19)	(2/10)

Heavy chain variable region HVR1 consensus sequence subgroup II showed the
 5 most sequence identity with the heavy chain variable region HVR1 of the E25 antibody.

Based on this comparison, the heavy chain variable region FR1 consensus sequence
 II was chosen to be imported into the E25 antibody sequence in order to determine whether
 identification of a FR1 sequence according to this method would improve folding and yield
 of the expressed antibody.

10

**B. Preparation of expression vectors encoding humanized anti-IgE antibody E25
 having human subgroup II framework sequences**

The heavy chain FR1 subgroup III sequence in the E25 antibody was substituted
 15 with a consensus sequence subgroup II to determine the effect of the substitution on the
 expression, folding and yield of the anti-IgE antibody E25.

Sequences of the heavy and light variable domains of E25 were subcloned into PRK
 plasmids as described in U.S. Patent No. 6,172,213 B1 for generation of full-length
 humanized anti-IgE molecules. The sequences for the heavy and light chains for the anti-
 20 IgE humanized antibody were subcloned into separate cistron vector as described in
 Example 2. The resulting plasmid, pE25-11, has a polynucleotide sequence (SEQ ID NO:
 20) encoding heavy and light chain variable domain amino acid sequences (SEQ ID NO:
 21), shown in Figure 20. The heavy chain FR1 sequences of E25 are according to subgroup
 III, except for change A24V made as part of humanization.

25 Replacement of the heavy chain FR1 subgroup III sequence with the subgroup II
 sequence, was performed as described previously. In order to change the FR1 subgroup III
 consensus to the subgroup II consensus sequence, 10 amino acid substitutions were
 required. The resulting plasmid, pE25-SGII, encodes heavy and light chain variable domain
 amino acid sequences shown in Figure 21.

30

C. Expression, Folding and Yield of Humanized anti-IgE Antibodies Having FR1 Consensus Subgroup Substitution

Expression constructs as described in Section B were transformed into bacteria to
5 determine expression, folding and yield of the E25 antibodies as described in Example 2. Non-reducing and reducing SDS-PAGE and immunoblot analysis of the resulting bacterial lysates was performed as described in Example 2, except that the samples were generated from fermentation cell paste, not from a shake flask induction. However, the cell lysates were obtained and processed as described previously for shake flask samples.

10

D. Results

Figure 5 shows the results of immunoblot analysis of lysates from induced cells transformed with pE25-11 (*E25HCFR1/SGIII*; lane 2), and pE25-SGII (*E25HCFR1/SGII*; lane 3). Figure 5A shows an immunoblot analysis of the whole cell lysates prepared under
15 reducing conditions and separated on a polyacrylamide gel, demonstrating that approximately equal amounts of heavy and light chains are produced from induced cells expressing pE25-11 (*E25HCFR1/SGIII*; lane 2), and pE25-SGII (*E25FR1/SGII*; lane 3). Figure 5B demonstrates that substitution of the heavy chain FR1 Subgroup III consensus sequence (+A24V) with the subgroup II consensus sequence results in increased yield of
20 assembled anti-IgE E25 antibody. This increased yield may be due to improved heavy chain folding efficiency since, regardless of which FR1 is used, the production of the individual heavy and light chains does not significantly change.

The relative yield of the completely disulfide bonded antibody products was determined by scanning densitometry of the gels as described in Example 2. The results
25 shown in Table 7 below indicate that replacement of the FR1 subgroup III residues with Subgroup II residues increased antibody yield in a fermentation sample about 2 fold. In all likelihood, the improvement would have been greater if pE25-11 did not have the A24V change (done as part of the humanization process; Ala is the SGIII consensus residue and V is the SGII consensus residue at position 24). This change probably improves the folding
30 and yield of the starting construct, pE25-11.

TABLE 7

anti-IgE (E25)		
5	<u>Plasmid</u>	<u>Yield</u>
	pE25-11 (FR1=SGIII)	1
	pE25-SGII (FR1=SGII)	1.2 (n=1)
	pE25-SGII (fermentation sample)	1.8 (n=1)
10	These results support the view that heavy chain FR1 sequences that provide for increased antibody folding, assembly and yield can be predicted by comparing the HVR1 region sequence of the antibody with the corresponding sequences in the human variable domain subgroup consensus sequences and identifying the subgroup with the most sequence identity to the HVR1 sequence of the antibody.	

15

Example 4

Heavy chain HVR1, HVR2 and HVR3 comparisons to human subgroup heavy chain variable domain consensus sequences.

To examine whether other HVR sequences could be used to select the human subgroup variable domain consensus sequence, a number of different antibodies were analyzed for sequence identity in HVR sequences with the corresponding sequences in the human subgroup variable domain consensus sequences. The heavy chain HVR1 (amino acids 26-35), HVR2 (kabat defined, amino acids 50-65) and HVR3 (kabat defined, amino acids 95-102) amino acid sequences of an antibody were aligned with each of the corresponding sequences of each of the human subgroup heavy chain variable domain consensus sequence and the % identity calculated as described previously. The results for each antibody are shown in Table 8 below.

30

TABLE 8

SEQUENCE SIMILARITY CHART

xVEGF (VNERK) Heavy Chain

	<u>HVR1</u>	<u>HVR2</u>	<u>HVR3</u>
Consensus Subgroup I	70%	44%	28%
Consensus Subgroup II	18%	28%	19%
Consensus Subgroup III	40%	26%	25%

xIgE (E25) Heavy Chain

	<u>HVR1</u>	<u>HVR2</u>	<u>HVR3</u>
Consensus Subgroup I	40%	33%	6%
Consensus Subgroup II	55%	50%	25%
Consensus Subgroup III	20%	47%	25%

xTF Heavy Chain

	<u>HVR1</u>	<u>HVR2</u>	<u>HVR3</u>
Consensus Subgroup I	20%	44%	22%
Consensus Subgroup II	18%	28%	19%
Consensus Subgroup III	40%	26%	25%

xCD40 Heavy Chain

	<u>HVR1</u>	<u>HVR2</u>	<u>HVR3</u>
Consensus Subgroup I	60%	44%	11%
Consensus Subgroup II	27%	28%	6%
Consensus Subgroup III	30%	26%	13%

5

The comparison shows that in 3 out of 4 cases, a comparison of the HVR1 and/or HVR2 sequences of an antibody variable domain resulted in the selection of the same human subgroup variable domain consensus sequence. In cases where the HVR1 and HVR2 differ in the identification of the human consensus subgroup with the most sequence identity, the consensus sequence with the most sequence identity to the HVR1 is preferably selected. In contrast, the HVR3 amino acid sequences tend to be very diverse in both sequence and in length when antibodies are compared to one another or to consensus sequences and therefore, were not useful in the selection of the human subgroup variable domain consensus sequence.

15

Example 5**Expression and Assembly of anti-VEGF VNERK
Antibodies with Single Amino Acid Substitution in FR1**

5

A. Preparation of Humanized anti-VEGF Antibody Expression Vectors Having Single Amino Acid Substitutions in FR1

Substitution of the FR1 heavy chain subgroup III residues with the FR subgroup I residues in anti-VEGF antibodies increased the yield of assembled antibody. Anti-VEGF antibodies were constructed with single amino acid substitutions at each of the FR1 subgroup III residues which were different than subgroup I residues at that position. The FR1 subgroup III residues were compared to FR subgroup I residues and where the amino acid differed at a position, the FR1 subgroup III residue was changed to the amino acid at the corresponding position in the subgroup I sequence. The antibodies with single amino acid substitutions were examined for folding efficiency and yield.

B. Preparation of Single Substitution Antibodies in Subgroup III Backbone.

The heavy chain FR1 subgroup III consensus sequence (SEQ ID NO: 3) was compared to heavy chain FR1 subgroup I consensus sequence (SEQ ID NO: 1) as shown below:

Subgroup III Sequence	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
	E	V	Q	L	V	E	S	G	G	G	L	V	Q	P	G	G	S	L	R	L	S	C	A	A	S
Subgroup I Sequence	Q					Q			A	E	V	K	K			A		V	K	V			K		

Single amino acid substitutions at each of residues 1, 6, 9, 10, 11, 12, 13, 16, 18, 19, 20 and 23 in the heavy chain FR1 were made one at a time to determine the effect of substitution of the subgroup I amino acid at each position. The antibodies are identified by the position and type of substitution made. For example, a substitution at the first residue of the glutamic acid residue in the subgroup III sequence for glutamine residue in the subgroup I sequence is designated E1Q. Other antibodies with substitutions are identified in a similar manner.

The vector designated pVKE6Q is a separate cistron vector prepared by modifying plasmid pxVG11NERK. Single amino acid substitutions were made by oligonucleotide mutagenesis as described previously. The heavy chain FR1 of VNERK was modified so that the glutamate at amino acid position 6 was converted to glutamine. Other single amino acid substitutions were made in a similar manner.

C. Expression, Folding and Yield of Humanized anti-VEGF FR1 with Single Amino Acid Substitutions

5 Anti-VEGF expression constructs were transformed into bacteria as described in Example 2 to determine expression, folding, and yield of the anti-VEGF VNERK antibodies having the indicated single amino acid substitutions in the heavy chain FR1 sequence. Lysates prepared under non-reducing and reducing conditions were separated by SDS-PAGE and immunoblot analysis of the resulting bacterial lysates was performed as
10 described in Example 2.

D. Results

Figure 6 shows the results of immunoblot analysis of lysates from cells expressing the antibodies with the single amino acid substitutions in FR1 of the anti-VEGF antibody as
15 described above. In each blot, the antibodies were compared to the wild type VNERK having the subgroup III FR1 sequence (lane 1) and the VNERK antibody having the subgroup I FR1 replacement (lane 8).

The results in Figure 6A and 6B show that when separated under reducing conditions, the antibodies with single substitutions produced approximately equivalent
20 amounts of heavy and light chains when compared to one another. The analysis of the anti-VEGF VNERK antibody lysates prepared under non-reducing conditions (Figures 6C and D) show that single amino acid substitutions at E6Q and A23K increased the yield of assembled anti-VEGF antibody about 2 fold ; E1Q, L11V, Q13K, L18V and R19K resulted in slight improvement or were neutral; and G9A, G10E, V12K, G16A and L20V resulted in
25 a decrease in the yield of assembled anti-VEGF antibody. (Figures 6C and 6D).

The relative yield of the single amino acid substitution antibodies relative to the unmodified VNERK antibody are shown in Table 9 below.

TABLE 9

	<u>Plasmid</u>	<u>Yield</u>
	pxVG11VNERK	1
5	E1Q	1.4 +/- 0.1 (n=4)
	E6Q	1.9 +/- 0.2 (n=5)
	G9A	band not detected (n=2)
	G10E	0.3 +/- 0.1 (n=2)
	L11V	1.5 +/- 0.0 (n=3)
10	V12K	0.1 +/- 0.0 (n=2)
	Q13K	1.1 (n=1)
	G16A	0.7 (n=1)
	L18V	1.1 (n=1)
	R19K	1.4 (n=1)
15	L20V	0.2 (n=1)
	A23K	2.1 +/- 0.2 (n=2)
	pVKFR1-2	2.2 +/- 0.2 (n=9)

A second series of samples was prepared under non-reducing conditions to compare yield of assembled products for the antibodies with single amino acid substitutions E1Q, E6Q, L11V and A23K with one another and the VNERK with unmodified subgroup I or subgroup III FR1 sequences. (Figure 7B).

The results show that the improved yield was primarily seen when changes were made at position 6 and position 23. Since each of these antibodies produced about equal amounts of heavy and light chains, the improved yield due to change at E6Q and A23K suggest that certain residues within the FR1 may have a greater influence on folding efficiency than others.

Example 6

Preparation of Expression Vectors Encoding Humanized anti-VEGF Antibodies with Substitutions at Residues Proximal to Variable Region Disulfide Bonded Cys Residues

An important structural feature of antibody variable domains is the intrachain variable domain disulfide bond connecting both of the B sheets formed in the antibody variable domain. Removal of the disulfide bond residues, especially in the variable heavy chain, results in a decrease in assembled products and an increase in aggregates (Ramon et al., *J. Mol. Biol.*, 290:535 (1999)). Typically, each variable domain has a single disulfide bond between a pair of cys residues found at conserved positions. Experiments were undertaken to determine whether any residue proximal to the disulfide bonded cys residues could also impact the folding and assembly of antibody molecules and whether one could predict which substitutions at those residues would improve antibody yield.

A. Identification of Amino Acid Positions Proximal to Disulfide Bonded Cys Residues

5 Positions in the antibody variable domain proximal to the disulfide bonded cys residues in the 3-dimensional structure of the antibody were identified by inspection of the crystal structure of humanized anti-VEGF variant Fab-12 (accession no. 1BJ1; Nucler et al., *Structure* 6:1153 (1998)) using the graphics program MIDAS (available from University of California, San Francisco). An amino acid position in a three-dimensional structure was
10 considered proximal to a cys residue that forms an intrachain disulfide bond when the side chain of the amino acid in that position (or for Gly, its alpha carbon) is about 5 angstroms or less from the cys residue or its side chain.

The amino acid positions proximal to the disulfide bonded cys residue for the anti-VEGF antibody variable domains were identified as follows:

15 In the light chain, the amino acid positions were 4, 6, 33, 35, and 71.

In the heavy chain, the amino acid positions were 4, 6, 34, 36, 78, and 104.

The anti-VEGF antibody, anti-TF antibody, anti-CD-18 antibody, and the 4D5 antibody all have framework regions from heavy chain consensus subgroup III. A crystal structure of each antibody was also analyzed using the program SOLV (G.S. Smith 1985,
20 "A computer program for the calculation of molecular volume and surface area of proteins", Merch, Sharpe and Dohme, Res. Laboratories, QCPE). Amino acid positions with a loss of about 10 square angstroms or greater of solvent accessible surface area by contacting the cys residue were selected. The same amino acid positions identified above were identified as positions proximal to disulfide bonded cys residues in each of the variable domains.

25

B. Construction of anti-VEGF Antibodies with Substitutions at Positions Proximal to Disulfide Bonded Cys Residues

Anti-VEGF antibodies were constructed with single or double mutations at one or
30 more of the amino acid positions identified as proximal to disulfide bonded cys residues in the 3-dimensional structure. The anti-VEGF antibody used to construct the modified antibodies is an anti-VEGF antibody identified as first generation wild type. The nucleotide (SEQ ID NO: 24) and amino acid sequence (SEQ ID NO: 25) of the heavy and light chain of anti-VEGF first generation wild type are shown in Figure 22. The starting construct is
35 designated pVG50. The antibodies constructed are identified below.

M4L	single substitution in the light chain
F71Y	single substitution in the light chain
M34I	single substitution in the heavy chain
E6Q; M34I	double substitution in the heavy chain
A78L	single substitution in the heavy chain

Amino acid residues at positions L33 and W35 in the light chain and L4, W36, and G104 in the heavy chain were not substituted because the amino acid residues in these positions are highly conserved across all subgroup consensus sequences and have the same amino acid at that position. Results from Example 5 show that the E6Q mutation greatly increased the yield of assembled product and was not tested as single mutation in this example.

The amino acid at position 78 in the first generation "wt" anti-VEGF antibody was changed from the consensus subgroup III residue leucine to the murine residue alanine at that position as a part of humanization of the antibody and to improve affinity of the antibody. Presta et al., Cancer Res., 57:4593-4599 (1997). Thus, the first generation wt antibody has an alanine at position 78 which corresponds to the amino acid found at that position in the subgroup I consensus sequence. The variant antibody A78L shown above has a substitution changing the subgroup I amino acid, Ala, of the first generation wt parent antibody back to the subgroup III amino acid, leucine at position 78.

Once the amino acid positions proximal to the cys residues were identified, single or double amino acid substitutions were made. The amino acid that was selected for substitution at each position was the amino acid found at the corresponding position in subgroup I sequence, except for position 78. The first generation wt anti-VEGF antibody heavy chain has a HVR1 sequence that has more sequence identity to consensus subgroup I HVR1 sequence than the subgroup III sequence. Thus, the amino acid substituted was the amino acid found at that position in the subgroup I sequence.

The anti-VEGF antibody Y0317 was also substituted at residue M34 in the heavy chain and F71 in the light chain. The M34 residue (subgroup III) was substituted with isoleucine (subgroup I). The F71 residue (subgroup III) was substituted with tyrosine.

Anti-VEGF antibody VNERK antibodies were constructed with the subgroup I amino acid at position 78 (A78), with the subgroup II amino acid at position 78 (F78), and the subgroup III amino acid at position 78 (L78).

The antibodies modified as described above were prepared by using the vectors described previously; anti-VEGF (VNERK) (Figure 15, SEQ ID NO: 5; and anti-VEGF Y0317 (Figure 16, SEQ ID NO: 6). The first generation wt anti-VEGF nucleotide and amino acid sequence is provided in Figure 22.

C. Expression, Folding and Yield of Humanized anti-VEGF with Substitutions at Positions Proximal to Disulfide Bonded Cys Residues

5 Expression constructs were transformed into bacteria to determine expression, folding and yield of the anti-VEGF antibodies as described in Example 2. Non-reducing and reducing lysates were separated by SDS-PAGE and immunoblot analysis of the resulting bacterial lysates was performed as described in Example 2.

D. Results of Immunoblot Analysis

Figure 8 shows the results of the immunoblot analysis of lysates from induced cells transformed with first generation wt VEGF antibody and the antibodies with amino acid substitutions at the following positions: M4L (light chain); F71Y (light chain); M34I (heavy chain); E6Q/M34I (heavy chain);. The results in Figure 8A demonstrate that approximately
 15 equal amounts of the heavy and light chains are produced when each antibody is compared to one another. In Figure 8B, the results show that the E6Q/M34I variant had greatly increased yield (about 16 fold) of completely disulfide bonded antibody product compared to the first generation wt control. The antibodies with mutations at positions 4 (about 2 fold) and 71 in the light chain (about 2.4 fold) and 34 (about 4.4 fold) in the heavy chain had
 20 an improvement in yield over the first generation wild type control. The results from the densitometry scans are shown below in Table 10.

TABLE 10

	<u>Plasmid</u>	<u>Yield</u>
25	pVG50 (1st gen wt)	1
	pVG50M4L (LC M4L)	1.9 +/- 0.2 (n=2)
	pVG50F71Y (LC F71Y)	2.4 (n=1)
	pVG50M34I (HC M34I)	4.4 +/- 0.1 (n=2)
	pVG50AA (M4L+M34I)	10.4 +/- 1.0 (n=3)
30	pVG50E6Q (HC E6Q +M34I)	16.0 (n=1)

When the residue at position 78 was changed from subgroup I residue back to the subgroup III residue, the antibody yield was reduced. Anti-VEGF VNERK antibodies with subgroup I amino acid A78 at position 78; subgroup II amino acid F78 at position 78; and
 35 subgroup III amino acid L78 were also tested for antibody yield. The results are shown in Figure 10. The anti-VEGF VNERK antibody having the subgroup I A78 residue displayed significantly higher assembled antibody yield (about 36 fold) compared to the antibody with the subgroup III (A78L) substitution at that position. The results of the scanning densitometry are shown in Table 11.

TABLE 11

	Plasmid	Yield
5	A78L -SGIII	1
	A78-SGI	36.0 (n=1)
	A78F-SGII	2.7 (n=1)

Thus, substitutions of amino acids in positions proximal to disulfide bonded cys residues with subgroup I amino acids improves antibody yield. Substitution back to subgroup III residues or subgroup II residue at position 78 reduced assembled antibody yield.

Another double mutant was constructed with a single amino acid substitution in each of the light chain and the heavy chain: M4L (light chain) and M34I (heavy chain). The yield of assembled antibody products was compared to the wt first generation anti-VEGF antibody and the M4L and M34I single substitution antibodies. Each antibody produced approximately equivalent heavy and light chains as seen in Figure 9A. The results in Figure 9B show that anti-VEGF with both substitutions had increase in assembled antibody yield about 10.4 fold over first generation wild type. (See Table 10). The anti-VEGF antibody with both substitutions also had an increased yield as compared with the antibodies with a single amino acid substitution at position 4 and position 34.

The effect of single substitutions in the light and heavy chain in antibody Y0317 was examined. Single amino acid substitution antibodies were constructed and tested for antibody yield. The antibodies had the following substitutions: M34I (heavy chain) and F71Y in the light chain. The results are shown in Figures 11 and 12. In Figure 11, the M34I change in the heavy chain increased antibody yield over the Y0317 antibody about 5 fold. The F71Y change in the light chain also increased antibody yield about 2.4 fold compared to Y0317, but not to the same extent as the M34I change. (Figure 12) The results of the scanning densitometry are shown in Table 12.

30 **TABLE 12**

	<u>Plasmid</u>	<u>Yield</u>
35	pXVG2AP11	1
	pY0M34I (HC M34I)	5.0 (n=1)
	pY0F71Y (LC F71Y)	2.4 (n=1)

In summary, we analyzed a number of different anti-VEGF antibodies and variants of the antibodies with one or more substitutions at amino acids in positions proximal to disulfide bonded cys residues. The results show that substitution of all of the amino acids in positions proximal to disulfide bonded cys residues with amino acids found at corresponding positions in the subgroup I consensus sequence in the heavy chain increased

antibody yield, likely through improved folding efficiency. These results suggest that prediction of the heavy chain subgroup FR based on HVR1 and/or HVR2 comparison may also predict which substitutions to make at positions proximal to the disulfide bonded cys residues to improve antibody folding and/or assembly and, therefore, yield.

5

Example 7

Expression and Yield of anti-VEGF VNERK Antibodies with FR2 and FR3 Substitutions

10 The anti-VEGF VNERK antibody was modified by additional changes to the framework regions of the heavy chain. The parent or wild-type anti-VEGF VNERK antibody was originally constructed with human heavy chain consensus sequences subgroup III for all of the framework regions in the heavy chain variable domain. The earlier studies demonstrated that replacement of the FR1 sequence of the heavy chain to the FR1 subgroup
15 I consensus sequence improved antibody yield. The subgroup I consensus sequence was selected as the consensus sequence subgroup with the greatest sequence identity in the HVR1 region to the HVR1 region of the anti-VEGF VNERK antibody. The next step addressed the question of whether substitution of additional heavy chain FRs with the subgroup I consensus sequence could further increase antibody yield.

20

A. Preparation of Expression Vectors Encoding Anti-VEGF Antibody with Modified FRs

Plasmid pVKFR1-2 prepared as described in Example 2 was used to make
25 additional anti-VEGF VNERK antibodies with modified framework regions. Plasmid pVKSGI.1.2 was prepared by modifying the sequence of plasmid pVKFR1-2 so that the FR2 amino acid sequence was changed from the subgroup III consensus sequence to the subgroup I consensus sequence using the method described in U.S. Patent No. 5,747,662. The change in the FR2 heavy chain sequence was in the region of amino acids 36-49. A
30 total of 2 amino acid substitutions were necessary to change the FR2 subgroup III consensus sequence to that of the subgroup I consensus sequence. The plasmid pVKSGI.1.2 encodes an anti-VEGF antibody with both the heavy chain FR1 and FR2 region residues having the sequence of human consensus sequence subgroup I.

Plasmid pVKSGI was prepared by further modifying plasmid pVKSGI.1.2.
35 Plasmid pVKSGI encodes an anti-VEGF heavy chain variable domain with FR1, FR2, and FR3 regions corresponding to that of the human consensus subgroup I sequence. The polynucleotide sequence (SEQ ID NO:26) and amino acid sequence (SEQ ID NO:27) of anti-VEGF antibody with all FR sequence of human consensus sequence subgroup I in

plasmid pVKSGI is shown in Figure 23. The amino acid sequence of FR4 is identical for all three heavy chain consensus subgroup sequences. The FR3 region of the anti-VEGF antibody includes amino acids 66 to 94 in the heavy chain variable domain. A total of 11 amino acid substitutions were necessary to change the FR3 region sequence of the parent antibody, pxVG11VNERK, from the consensus sequence subgroup III sequence, including changes made to this region as a part of the humanization process, to that of the subgroup I sequence.

B. Expression, Folding and Yield of Modified Anti-VEGF Antibodies

Expression vectors described in Section A were transformed into bacteria to determine expression, folding and yield as described in Example 2.

C. Immunoblot Analysis

Soluble fractions of each sample were prepared as follows: (1) a 5 O.D.₆₀₀ pellet of each sample was resuspended in 225 ul of 50 mM NaCl + 5 mM EDTA + 50 mM Tris pH 8 + 1 mg/ml lysozyme; (2) 25 ul of 100 mM IAA (Iodoacetic acid; Sigma I-2512) was then added; (3) the cell suspensions were vortexed and lysed by sonicating for 2 x 2 minutes at 50% pulse (Sonics & Materials, Inc., Danbury, CT) (the samples were kept in an ice water bath during sonication to dissipate the heat generated during the process); (4) the samples were centrifuged for 5 minutes in a microfuge; (5) 100 ul of each supernatant (soluble fraction) was then acetone precipitated by adding approximately 500 ul of acetone to each sample and leaving the samples at RT approximately 15 minutes; (6) each precipitate was resuspended in 50 ul of dH₂O + 50 ul of 2X sample buffer; (7) the samples were heated at about 90 C for 3-5 minutes, vortexed well, allowed to cool to RT; and, (8) the samples were centrifuged again for 5 minutes and the supernatants were transferred to clean eppendorf tubes.

The soluble fractions were then loaded (5-10 ul in each well) onto a 10 well, 1.0 mm NOVEX manufactured 12% Tris-Glycine SDS-PAGE and electrophoresed at about 120 volts for 1.5-2 hours. The resulting gels were either stained with Coomassie Blue or used for an immunoblot.

For the immunoblot, the gel was transferred and treated as previously described in Example 2 except a different detecting antibody was used. The antibody used for detection in this experiment was an anti-Fc antibody (Bethyl Laboratories, Inc.; Goat anti-Human IgG-Fc Fragment HRP conjugated #A80-104P) diluted 1:500,000. Relative yields were calculated using scanning densitometry as previously described.

D. Results

The effect of substitution of additional FR residues on antibody yield was examined. An antibody was constructed with both FR1 and FR2 region residue changes from human consensus subgroup III residues to the human consensus subgroup I residues.

5 An antibody was also constructed with FR1, FR2, and FR3 changed to human consensus subgroup I. The results of the antibody yield are shown in Figure 13. Figure 13A shows a gel stained with Coomassie Blue and Figure 13B shows the immunoblot results using an anti-Fc antibody. The results show that changing the FR sequences from the subgroup III consensus sequence to the subgroup I consensus sequence increased antibody yield.

10 Scanning densitometry results are shown in Table 13.

TABLE 13

	Plasmid	Yield	
		Coomassie Gel	antiFc Blot
15	pxVG11VNERK (SGIII, except for humanization changes)	Band not detected	1
	pVKFR1-2 (FR1=SGI)	1*	2.8
	pVKSGI.1.2 (FR1+FR2=SGI)	1.9	3.2
	pVKSGI (FR1+FR2+FR3=SGI)	2.6	3.6

20

*Since the band for pxVG11VNERK was not detectable by Coomassie Blue staining, data was normalized to pVKFR1-2.

These results suggest that making changes in more than one framework region provides a greater increase in antibody yield over the increase demonstrated by changing only one framework region. Substitution of all three framework region residues with the selected subgroup consensus sequence resulted in about a 3.5 fold increase in antibody yield compared to the parent antibody anti-VEGF VNERK. This increase in antibody yield is most likely due to increased folding efficiency of the heavy chain. Since anti-VEGF VNERK is a humanized antibody, some of the FR region substitutions at the positions identified in accord with the methods of the invention were already made to improve antigen binding affinity. As a result, the improvement in yield seen with the subgroup I constructs may have been greater than about 3.5 fold if the changes to the starting subgroup III sequence had not already been made. The increase in yield may have been greater if the changes were made prior to humanization, i.e. starting with the chimeric xVEGF instead of the humanized xVEGF thereby going from a purely SGIII consensus sequence to a SGI consensus sequence in all four FRs. Additionally, starting with the chimeric antibody and

25

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making the substitutions to SGI, rather than SGIII, may have also significantly shortened the time required for humanization.

Example 8

5 **Expression and Secretion of anti-VEGF antibodies from CHO cells**

 In prokaryotic cell cultures, FR1 substitution of the subgroup III heavy chain with subgroup I heavy chain sequences in anti-VEGF antibodies increased assembled antibody yield. The anti-VEGF antibodies first generation wild-type with HC M34I, VNERK, and
10 YO317 were tested to compare the effect of the same FR1 substitution on antibody secretion in mammalian cells. Specifically, the heavy chain FR1 sequence from Subgroup III was substituted with the heavy chain FR1 sequence from Subgroup I. Secretion levels of fully assembled antibody from a mammalian Chinese hamster ovary (CHO) cell line were measured by ELISA and by densitometry of immunoblots.

15

A. **Preparation of Expression Vectors Encoding Anti-VEGF Antibodies with Modified FRs**

 The heavy chain constructs were subcloned from the prokaryotic expression
20 vectors, as described in Examples 2 and 6 into mammalian production vector SV40.PD. The vector contains a Simian Virus 40 (SV40) promoter and a puromycin and dihydrofolate reductase (DHFR) fusion gene for selection. In addition, the mammalian signal sequences used for antibody production were cloned into the vector. For this experiment, there were six different heavy chain constructs--1st Generation xVEGF SGI
25 with HC M34I, 1st Generation xVEGF SGIII with HC M34I, YO317 xVEGF SGI, YO317 xVEGF SGIII, and VNERK xVEGF SGI, and VNERK xVEGF SGIII.

 In parallel, the light chain constructs from the prokaryotic expression vectors were subcloned into a pRK expression vector containing a Cytomegalovirus (CMV) promoter. The two light chain constructs were the M4 xVEGF light chain (normally coexpressed with
30 the first generation wild-type xVEGF heavy chain) and the L4 xVEGF light chain (normally coexpressed with the YO317 and VNERK xVEGF heavy chain). The light chain expression vectors also contained the aforementioned mammalian signal sequences used for antibody production.

B. Expression, Folding, and Secretion of Modified Anti-VEGF Antibodies**1. Transfection**

The heavy chain constructs were co-transfected with either the M4 or L4 light chain constructs. Plasmids were transiently transfected into DP12 cells. DP12 cells have been
5 described in U.S. Patent No. 6,673,580, which is hereby incorporated by reference

2. ELISA

ELISA methods for quantitating full length antibody are known to those of skill in the art and have been published in U. S. Patent Application Publication 20030190317.
10

3. Immunoblot

Immuno blot analysis was performed according to methods well known in the art.

C. Results

The previously disclosed examples demonstrated that constructs with SGI FR1
15 consensus sequences had improved antibody yields over constructs with SGIII in the prokaryotic system. The yield of VNERK xVEGF with a SGI FR1 consensus sequence was 2.2 ± 0.2 -fold higher than VNERK constructs containing a SGIII FR1 consensus sequence. The yield of YO317 xVEGF with a SG1 FR1 consensus sequence was 8.2 ± 1.9 -fold higher
20 than YO317 constructs with a SGIII FR1 consensus sequence. Similarly, the 1st generation wild-type xVEGF had higher yields when SGI substitutions were made in the heavy chain at positions proximal to disulfide bonded cysteine residues. Whether the higher yields could be replicated in the mammalian cell culture system was tested.

The amounts of fully assembled antibodies secreted were measured by ELISA four
25 days post-transfection. In the CHO cells, two of the three constructs with SGI FR1 consensus sequences had increased yield and secretion of assembled antibodies. The VNERK construct containing SGI sequence had approximately a 3.6 fold increase in secretion above the VNERK construct containing SGIII sequence. Similarly, the YO317 construct containing SGI sequence had approximately a 2.2 fold increase in secretion above
30 the YO317 construct containing SGIII sequence. However, the 1st generation wild-type construct with M34I had approximately a 3.8-fold decrease in secretion when the SGI FR1 consensus sequence was substituted for the SGIII FR1 sequence. Coexpression of the M4 or L4 light chain did not affect the secretion levels of a particular construct. Immunoblot analysis of the CHO cell culture media and cell lysates confirmed the ELISA results. (data
35 not shown)

Table 14

<u>Antibody Construct</u>	<u>Relative Secretion</u>
VNERK SG1:VNERK SG3	3.6 : 1.0
YO317 SG1:YO317 SG3	2.2 : 1.0
1st Gen SG1:1st Gen SG3	1.0 : 3.8

It is expected that some variation in results may occur when experiments are repeated. When the experiment was repeated, some differences were seen in the results but the trend was the same. The VNERK construct containing SGI sequence and the YO317 construct containing SGI sequence had increased yield and anti-VEGF 1st generation containing the SGI sequences had decreased yield.

The above results demonstrate that some antibodies modified with SGI sequences in accord with the methods of the invention are secreted from mammalian cells in greater quantities than antibodies containing SGIII sequences. Constructs modified in accord with the methods of the invention yield greater quantities of completely assembled antibodies in both the prokaryotic and mammalian cell cultures.

Although the foregoing refers to particular embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may be made to the disclosed embodiments without changing from the overall concept of the invention. All such modifications are intended to be within the scope of the invention.

We claim:

1. A method for producing an antibody or antigen binding fragment in high yield in cell culture, comprising:
 - a) expressing a variable domain of the antibody or antigen binding fragment comprising at least one modified FR in a host cell, wherein the modified FR has a substitution of at least one amino acid position with a different amino acid, wherein the different amino acid is the amino acid found at the corresponding FR position of a human subgroup variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 sequence of the variable domain, wherein the antibody or antigen binding fragment variable domain comprising the modified FR has improved yield in cell culture compared to an unmodified antibody or antigen binding fragment; and
 - b) recovering the antibody or antigen binding fragment variable domain comprising the modified framework from the host cell.
2. The method according to claim 1, wherein the antibody or antigen binding fragment is selected from the group consisting of a humanized antibody, a chimeric antibody, a monoclonal antibody, a human antibody, a multispecific antibody, diabodies, or an antibody generated by phage display.
3. The method according to claim 2, wherein the antigen binding fragment is a Fab fragment, F(ab')₂ fragment, scFV fragment, or sc(Fv)₂ fragment, a single arm antibody or single chain antibody.
4. The method according to any of claims 1-3, wherein the antibody is an anti-VEGF antibody or an anti-IgE antibody.
5. The method according to any of claims 4, wherein the antibody is a humanized antibody.
6. The method of any of claims 1 to 5, wherein expressing a variable domain of the antibody or antigen binding fragment comprising at least one modified FR in a host cell comprises expressing a polynucleotide encoding the variable domain comprising the at least one modified FR.

7. The method of claim 6, wherein the polynucleotide further comprises a polynucleotide encoding a constant region domain connected to the polynucleotide encoding the variable domain with modified FR to form a polynucleotide encoding a full-length heavy or light chain.
8. The method of claim 7, wherein the polynucleotide further comprises an expression vector.
9. The method of claim 7 or 8, further comprising recovering a full-length heavy or light chain or both from the culture.
10. The method according to any of claims 1- 9, wherein the host cell is a prokaryotic host cell.
11. The method according to any of claims 1-9, wherein the host cell is a mammalian cell.
12. The method according to any of claims 1-11, wherein the variable domain is a heavy chain variable domain or a light chain variable domain.
13. The method according to claim 12, wherein the HVR1 amino acid sequence is GYTFTNYGIN (SEQ ID NO: 14), GYDFTHYGMN (SEQ ID NO:18), or GYSITSGYSWN (SEQ ID NO:19) .
14. The method according to any of claims 1-13, wherein the framework region is selected from the group consisting of FR1, FR2, FR3, FR4 and mixtures thereof.
15. The method according to claim 14, wherein the human subgroup FR consensus sequence is a heavy chain FR1 sequence with a sequence selected from the group consisting of SEQ. ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3.
16. The method according to any of claims 1-15, wherein the yield of the antibody or antigen binding fragment comprising the modified FR is improved at least 2 fold compared to the unmodified antibody or antigen binding fragment.

17. The method according to claim 16, wherein the yield of the antibody or antigen binding fragment comprising the modified FR is improved at least 2 fold to 16 fold compared to the unmodified antibody or antigen binding fragment.
18. The method of any of claims 1-17, wherein at least two amino acid positions in at least one FR that have a different amino acid are substituted with the amino acids in the corresponding positions of the selected subgroup consensus sequence.
19. The method of claim 18, wherein the FR is a heavy chain FR1 and one of the amino acid positions is position 6 or position 23 or both, and the other position is selected from the group consisting of position 1, 11, 13, 18, 19, and mixtures thereof.
20. The method of claim 18 wherein amino acid positions 6 and 23 are substituted.
21. The method of claim 19, wherein all of the amino acid positions at positions 1, 6, 11, 13, 18, 19, and 23 of the heavy chain FR1 are substituted.
22. The method of any of claims 1-21, wherein all of the amino acid positions in a FR that have a different amino acid are each substituted with the amino acid in the corresponding FR position in the selected subgroup consensus sequence.
23. The method of claim 22, wherein the FR is FR1, FR2, or FR3.
24. The method of any of claims 1-23, wherein all of the amino acid positions that have a different amino acid in all FR are each substituted with the amino acid in the corresponding FR position in the selected subgroup consensus sequence.
25. A method for preparing a humanized antibody or antigen binding fragment, comprising:
- a) expressing a variable domain comprising at least one FR sequence from a selected human subgroup variable domain consensus sequence, and a HVR1 and/or HVR2 sequence of a non-human antibody in a host cell, wherein the selected human subgroup consensus sequence is the human subgroup consensus sequence that has a HVR1 and/or HVR2 sequence that has the most sequence identity to the HVR1 and/or HVR2 of the non-human antibody;
 - b) recovering the antibody variable domain from the host cell.

26. The method according to claim 25, wherein the variable domain is a heavy chain variable domain.
27. The method according to claim 25, wherein the variable domain is a light chain variable domain.
28. The method according to claim 26, wherein the HVR1 amino acid sequence is GYTFTNYGIN (SEQ ID NO: 14), GYDFTHYGMN (SEQ ID NO:18), or GYSITSGYSWN (SEQ ID NO:19) .
29. The method according to any of claims 25-28, wherein the FR is selected from the group consisting of FR1, FR2, FR3, FR4 and mixtures thereof.
30. The method according to claim 29, wherein the human subgroup FR consensus sequence is a heavy chain FR1 sequence with a sequence selected from the group consisting of SEQ. ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3.
31. The method of any of claims 25-30, wherein the variable domain comprises all of the FR of the selected human subgroup consensus sequence.
32. The method of any of claims 25-31, wherein the humanized antibody or antigen binding fragment has improved yield when produced in cell culture as compared to a or antigen binding fragment having the same HVR1 and/or HVR2 but without the selected FR.
33. The methods of any of claims 25-32, wherein expressing comprises: expressing an expression vector comprising a polynucleotide encoding a variable domain comprising the HVR1 and/or HVR2 of the non-human antibody, and the selected FR.
34. The method of claim 33, wherein the expression vector further comprises a polynucleotide encoding a constant domain connected to the polynucleotide encoding the variable domain to form a polynucleotide encoding a full-length heavy or light chain.
35. The method of claim 34, further comprising recovering the full-length heavy or light chain or both from the culture.

36. The method according to any of claims 25-35, wherein the host cell is a prokaryotic host cell.

37. The method according to any of claims 25-35, wherein the host cell is a mammalian cell.

38. A method for improving the yield of an antibody or antigen binding fragment in cell culture, comprising:

expressing a heavy chain variable domain of the antibody or antigen binding fragment comprising at least one modified FR in a host cell, wherein the modified FR has a substitution of at least one amino acid position in at least one FR with a different amino acid, wherein the different amino acid is the amino acid found at the corresponding FR position of a human heavy chain subgroup variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 sequence of the heavy chain variable domain, wherein the antibody or antigen binding fragment with the modified FR of the heavy chain has improved yield in cell culture compared to an unmodified parent antibody or antigen binding fragment; and recovering the antibody or antigen binding fragment variable domain comprising the modified framework from the host cell.

39. A method for improving the yield of an antibody or antigen binding fragment in cell culture, comprising:

modifying said at least one FR sequence of a variable domain of the antibody or antigen binding fragment such that it is at least 50% identical in sequence to the corresponding FR sequence of a selected subgroup consensus sequence to form a modified FR, wherein the modified FR has a substitution of at least one amino acid position with a different amino acid, wherein the different amino acid is the amino acid found at the corresponding FR position of the selected human subgroup variable domain consensus sequence, wherein the selected human subgroup consensus sequence has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 sequence of the variable domain, wherein the antibody or antigen binding fragment with the modified FR has improved yield in cell culture compared to an unmodified parent antibody or antigen binding fragment; and recovering the variable domain with the modified FR from the host cell.

40. The method according to claims 38 or 39, wherein the HVR1 amino acid sequence is GYTFTNYGIN (SEQ ID NO: 14), GYDFTHYGMN (SEQ ID NO: 18), or GYSITSGYSWN (SEQ ID NO: 19).

41. The method of any of claims 38-40, wherein at least two amino acid positions that have a different amino acid in at least one FR are substituted with amino acids in the corresponding position of the selected subgroup consensus sequence.

42. The method of claim 41, wherein amino acid positions 6 and 23 of heavy chain FR1 are substituted.

43. The method of claim 41, wherein amino acid positions 1, 6, 11, 13, 18, 19 and 23 of the heavy chain FR1 are substituted.

44. The method according to any of claims 38, wherein expressing comprises expressing an expression vector comprising a first polynucleotide that encodes a variable domain comprising the HVR1 and/or HVR2 amino acid sequence of the antibody or antigen binding fragment and at least one modified FR.

45. The method according to claim 44, wherein the expression vector further comprises a second polynucleotide encoding a constant domain, wherein the first and second polynucleotide are operably linked to a promoter; a heat stable enterotoxin sequence that can direct secretion to the periplasm; and a terminator sequence.

46. The method according to any of claims 35-45, wherein the host cell is a prokaryotic host cell.

47. The method according to any of claims 35-45, wherein the host cell is a mammalian cell.

48. The method according to claim 39, wherein the step of modifying comprises substituting all of the FRs of the variable domain with each of the corresponding FRs of the selected subgroup.

49. The method according to any of claims 38-48, wherein the framework region sequence is selected from the group consisting of FR1, FR2, FR3, FR4 and mixtures thereof.

50. A method for producing an antibody or antigen binding fragment in high yield in cell culture comprising:

- a) expressing a modified variable domain of the antibody or antigen binding fragment in a host cell, wherein the modified variable domain has a substitution of at least one amino acid position proximal to a cys residue that participates in an intrachain variable domain disulfide bond with a different amino acid, wherein the different amino acid is the amino acid found at corresponding position of a human subgroup variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 amino acid sequence of the variable domain, wherein the antibody or antigen binding fragment comprising the modified variable domain has improved yield in cell culture compared to the antibody or antigen binding fragment; and
- b) recovering the antibody or antigen binding fragment comprising the modified variable domain from the host cell.

51. The method according to claim 50, wherein the variable domain is a heavy chain variable domain or a light chain variable domain.

52. The method according to claim 51, wherein said at least one position is selected from the group consisting of the amino acid position 4 of the light chain, the amino acid position 6 of the light chain, the amino acid position 33 of the light chain, the amino acid position 35 of the light chain, the amino acid position 71 of the light chain and mixtures thereof.

53. The method according to claim 51, wherein said at least one position is selected from the group consisting of the amino acid position 4 of the heavy chain, the amino acid position 6 of the heavy chain, the amino acid position 34 of the heavy chain, the amino acid position 36 of the heavy chain, the amino acid position 78 of the heavy chain, the amino acid position 104 of the heavy chain and mixtures thereof.

54. The method according to claim 51, wherein said at least one position selected from the group consisting of amino acid position 4 of the light chain, amino acid position 6 of the light chain, amino acid position 33 of the light chain, amino acid position 35 of the light chain, amino acid position 71 of the light chain, and at least one position is selected from the group consisting of amino acid position 4 of the heavy chain, amino acid position 6 of the heavy chain, amino acid position 34 of the heavy chain, amino acid

position 36 of the heavy chain, amino acid position 78 of the heavy chain, and amino acid position 104 of the heavy chain.

55. The method according to any one of claims 50-54, wherein the at least one amino acid position is an amino acid position adjacent to the cys residue that forms an intra chain variable domain disulfide bond.

56. The method according to claim 55, wherein the at least one amino acid position is selected from group consisting of amino acid position 21, amino acid position 22, amino acid position 24, amino acid position 25, amino acid position 86, amino acid position 87, amino acid position 89 and amino acid position 90 in a light chain variable domain.

57. The method according to claim 55, wherein the at least one amino acid position is selected from the group consisting of amino acid position 20, amino acid position 21, amino acid position 23, amino acid position 24, amino acid position 90, amino acid position 91, amino acid position 93 and amino acid position 94 in a heavy chain variable domain.

58. The method according to any of claims 50-57, wherein the variable domain is from an anti-VEGF antibody or anti-IgE antibody.

59. The method according to any of claims 50-58, wherein the variable domain is from a humanized antibody or antigen binding fragment.

60. The method according to any of claims 50-59, wherein the step of expressing comprises expressing an expression vector comprising a first polynucleotide that encodes the modified variable domain sequence with an amino acid substitution in at least one of the amino acids proximal to a cys residue, wherein at least one amino acid is substituted with the amino acid at the corresponding position in the selected subgroup consensus sequence.

61. The method according to claim 50, wherein the expression vector comprises a second polynucleotide encoding antibody constant region domains, wherein the first and second polynucleotide are operably linked to a promoter; a heat stable enterotoxin sequence that can direct secretion to the periplasm; and a terminator sequence.

62. The method according to claim 61, further comprising recovering a full-length heavy or light chain or both.

63. The method according to any of claims 60-62, wherein the heavy chain variable domain has a substitution in amino acid position 4, 6, 34, 78, or mixtures thereof.

64. The method of any of claims 60-63, wherein the light chain variable domain has a substitution in amino acid position 4, 71, or mixtures thereof.

65. The method according to any of claims 50-64, wherein the host cell is a prokaryotic host cell.

66. The method according to any of claims 50- 64, wherein the host cell is a eukaryotic host cell.

67. The method according to any of claims 50-66, wherein the antibody or antigen binding fragment with modified variable domain has increased yield of at least 2 fold when produced in cell culture as compared to the antibody or antigen binding fragment.

68. The method according to claim 67, wherein the yield of the antibody or antigen binding fragment with the modified variable domain is increased at least 2 to 16 fold as compared to the antibody or antigen binding fragment.

69. The method of claim 50 further comprises:

- a) identifying at least one amino acid position in a second variable domain of the antibody or antigen binding fragment that is proximal to a cysteine residue that forms an intrachain variable domain disulfide bond in the second variable domain;
- b) selecting a variable domain subgroup consensus sequence having the most sequence identity with a HVR1 and/or HVR2 amino acid sequence of the second variable domain; and
- c) determining whether the amino acid in the amino acid position identified in the second variable domain is different than the amino acid in the selected subgroup consensus sequence; and
- d) placing at said at least one position in the second variable domain the amino acid found at the corresponding position in the selected subgroup consensus sequence to form a modified variable domain.

70. The method of claim 69, wherein the variable domain is a heavy chain variable domain and the second variable domain is a light chain variable domain.

71. A method for preparing a humanized antibody or antigen binding fragment, comprising:

- a) expressing a variable domain in a host cell, wherein the variable domain is formed by substituting at least one amino acid position proximal to a cys residue that participates in an intrachain variable domain disulfide bond with a different amino acid, wherein the different amino acid is the amino acid found at corresponding position of a human subgroup variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 amino acid sequence of the variable domain; and
- b) recovering the antibody variable domain from the host cell.

72. The method according to claim 71, wherein the variable domain is a heavy chain variable domain.

73. The method according to claim 71, wherein the variable domain is a light chain variable domain.

74. A method for improving the yield of an antibody or fragment thereof, comprising:

- a) identifying at least one amino acid position in a heavy chain variable domain that is proximal to a cys residue that participates in an intrachain disulfide bond in the heavy chain variable domain;
- b) selecting a first human antibody heavy chain variable domain subgroup consensus sequence having the most identity with a HVR1 and/or HVR2 amino acid sequence of the heavy chain variable domain; and
- c) placing at said at least one position in the heavy chain variable domain an amino acid found at the corresponding position in the selected first subgroup;
- d) identifying at one amino acid position in a light chain variable domain that is proximal to a cys residue that participates in an intrachain disulfide bond in the light variable domain;

- e) selecting a second human antibody light chain variable domain subgroup consensus sequence having the most sequence identity with a HVR1 and/or HVR2 amino acid sequence of the light chain variable domain; and
- f) placing at said at least one position in the light chain variable domain an amino acid found at the corresponding position in the second selected subgroup.

75. A polypeptide comprising a first variable domain comprising:

- a) at least one amino acid position that is proximal to a cys residue that forms an intrachain disulfide bond in the first variable domain;
- b) said at least one position having an amino acid found at a corresponding position of a human antibody subgroup consensus sequence selected for the most sequence identity with a HVR1 and/or HVR2 amino acid sequence of the first variable domain.

76. The polypeptide of claim 75, wherein the first variable domain is a heavy chain and the positions are amino acid positions 4, 6, 34, 78 or mixtures thereof.

77. The polypeptide of claim 75, wherein the first variable domain is a light chain and the amino acid positions are 4, 71, or both.

78. A polypeptide comprising a first and second variable domain comprising:

- a) a first variable domain with at least one amino acid position that is proximal to a cys residue that forms an intrachain disulfide bond in the first variable domain;
- b) said at least one position having an amino acid found at a corresponding position of a human antibody subgroup consensus sequence selected for the most identity with a HVR1 and/or HVR2 amino acid sequence of the first variable domain;
- c) a second variable domain with at least one amino acid position that is proximal to a cys residue that forms an intrachain disulfide bond in the second variable domain;
- d) said at least one position having an amino acid at a corresponding position of a human antibody subgroup consensus sequence selected for the most identity with a HVR1 and/or HVR2 amino acid sequence of the second variable domain; and wherein the first variable domain is a heavy chain and the second variable domain is a light chain.

79. An antibody comprising: a heavy chain variable domain comprising a HVR1 and at least one FR, wherein the FR has an amino acid residue of the corresponding FR of a human subgroup consensus sequence with the most sequence identity to the HVR1 and/or HVR2 amino acid sequence in at least two amino acid positions selected from the group consisting of 1, 6, 11, 13, 18, 19, 23, and mixtures thereof.

80. The antibody according to claim 79, wherein each of the amino acid positions has an amino acid of the corresponding position in FR of the human subgroup consensus sequence with the most sequence identity to the HVR1.

81. The antibody according to any of claims 78-80, wherein amino acid positions 6 and 23 each has an amino acid found at the corresponding position in the FR of a human subgroup consensus sequence with the most sequence identity to the HVR1.

82. A method for improving the yield of antibody or antigen binding fragments in cell culture, comprising:

expressing a variable domain of the antibody or antigen binding fragment comprising at least one modified FR in a host cell, wherein the modified FR is obtained by substituting at least one amino acid in a least one FR of a parent variable domain of the antibody or antigen binding fragment with a different amino acid, wherein the different amino acid is an amino acid found at the corresponding FR position of a human subgroup variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 amino acid sequence of the parent variable domain to form a modified FR, wherein antibody or antigen binding fragment comprising the modified FR has improved yield in cell culture compared to the parent antibody or antigen binding fragment; and recovering the antibody or antigen binding fragment comprising at least one modified FR from the host cell.

83. The method according to claim 82, wherein the parent variable domain is obtained from an antibody is selected from the group consisting of a humanized antibody, a monoclonal antibody, a human antibody, a multispecific antibody, diabodies, or an antibody generated by phage display.

84. The method according to claim 83, wherein the parent variable domain is from an antigen binding fragment that is a Fab fragment, F(ab')₂ fragment, scFV fragment, or sc(Fv)₂ fragment, single arm antibody, or single chain antibody.

85. The method according to any of claims 82-84, wherein the parent antibody variable domain is from an anti-VEGF antibody or anti-IgE antibodies.

86. The method according to any of claims 82-85, wherein the parent antibody variable domain is from a humanized antibody.

87. The method according to any of claims 82-86, wherein expressing comprises expressing an expression vector comprising a first polynucleotide that encodes the variable domain comprising the HVR1 and/or HVR2 amino acid sequence and the modified FR.

88. The method according to any of claims 82-87, wherein the HVR1 amino acid sequence is GYTFTNYGIN (SEQ ID NO: 14), GYDFTHYGMN (SEQ ID NO: 18, or GYSITSGYSWN (SEQ ID NO: 19).

89. The method according to any of claims 82-88, wherein the host cell is a prokaryotic host cell.

90. The method according to any of claims 82-89, wherein the variable domain is a heavy chain variable domain or light chain variable domain or both.

91. The method according to any of claims 82-90, wherein the host cell is a mammalian cell.

92. The method according to any of claims 82-91, wherein the FR is selected from the group consisting of FR1, FR2, FR3, FR4 and mixtures thereof.

93. The method according to any of claims 82-91, wherein the human subgroup FR consensus sequence is a FR1 sequence with a sequence selected from the group consisting of SEQ. ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3.

94. The method according to any of claims 82-93, wherein the yield of the antibody or antigen binding fragment with the modified FR is improved at least 2 fold compared to the antibody or antigen binding fragment with the parent variable domain.

95. The method according to any of claims 94, wherein the yield of the antibody or antigen binding fragment with the modified FR is improved at least 2 fold to 16 fold.

96. A method for improving the yield of antibody or antigen binding fragments in cell culture, comprising:

- a) expressing a polynucleotide encoding a variable domain of the antibody or antigen binding fragment comprising at least one modified FR in a host cell, wherein the modified FR has a substitution of at least one amino acid in the at least one FR with a different amino acid, wherein the different amino acid is the amino acid found at the corresponding FR position of a human subgroup variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 sequence of the variable domain, wherein the antibody or antigen binding fragment comprising the variable domain with the modified FR has improved yield in cell culture compared to an antibody or antigen binding fragment with an unmodified variable domain; and
- b) recovering the antibody or antigen binding fragment comprising the modified FR from the host cell.

97. The method according to claim 96, wherein the polynucleotide is an expression vector that comprises a polynucleotide encoding a variable domain comprising the modified FR and at least one constant region domain operably linked to a promoter, a heat stable enterotoxin sequence that can direct secretion to the periplasm, and a terminator sequence.

98. The method according to claim 96 or 97, wherein the host cell is a prokaryotic host cell.

99. The method according to claim 96 or 97, wherein the host cell is a eukaryotic host cell.

100. A method for improving the yield of antibody or antigen binding fragments in cell culture, comprising:

- expressing a polynucleotide molecule encoding a modified variable domain of a parent antibody or antigen binding fragment in a host cell, wherein the modified variable domain has a substitution of at least one amino acid proximal to a

cys residue that participates in an intrachain variable domain disulfide bond with a different amino acid, wherein the different amino acid is the amino acid found at corresponding position of a human subgroup variable domain consensus sequence that has a HVR1 and/or HVR2 amino acid sequence with the most sequence identity with a corresponding HVR1 and/or HVR2 amino acid sequence of the parent variable domain, wherein the antibody or antigen binding fragment comprising the modified variable domain has improved yield in cell culture compared to the parent antibody or antigen binding fragment; and

recovering the antibody or antigen binding fragment comprising the modified variable domain from the host cell.

101. The method according to claim 100, wherein the polynucleotide molecule comprises an expression vector that comprises a polynucleotide molecule encoding the modified variable domain and at least one constant region domain operably linked to a promoter, a heat stable enterotoxin sequence that can direct secretion to the periplasm, and a terminator sequence.

102. The method according to claim 100 or claim 101, wherein the host cell is a prokaryotic host cell.

103. The method according to claim 100 or claim 101, wherein the host cell is a eukaryotic host cell.

104. A method for improving the yield of antibody or antigen binding fragments in cell culture, comprising:

a) comparing a HVR1 and/or HVR2 amino acid sequence of a variable domain of a parent antibody or antigen binding fragment to a corresponding HVR1 and/or HVR2 amino acid sequence of each human subgroup variable domain consensus sequences and selecting a human subgroup variable domain consensus sequence that has the most sequence identity with the HVR1 and/or HVR2 sequence of the variable domain;

b) identifying at least one amino acid position in at least one FR in the variable domain selected from the group consisting of FR1, FR2, FR3, FR4 and mixtures thereof, wherein the amino acid position has a different amino acid than the amino acid at a corresponding position of the selected human subgroup variable domain consensus sequence; and

c) substituting the at least one amino acid position identified in step (b) with the amino acid in the corresponding position of the selected human subgroup variable domain consensus sequence to form a variable domain with a modified FR;

wherein the antibody or antigen binding fragment with the modified FR has improved yield in cell culture compared to the parent antibody or antigen binding fragment.

105. The method according to claim 104, wherein the parent antibody is selected from the group consisting of a humanized antibody, a chimeric antibody, a monoclonal antibody, a human antibody, a multispecific antibody, diabodies, or an antibody generated by phage display.

106. The method according to claim 105, wherein the parent antigen binding fragment is a Fab fragment, F(ab')₂ fragment, scFV fragment, or sc(Fv)₂ fragment, single arm antibody, or single chain antibody.

107. The method according to any of claims 104-106, wherein the parent antibody is an anti-VEGF antibody or an anti-IgE antibody.

108. The method according to claim 107, wherein the parent antibody is a humanized antibody.

109. The method of any of claims 104 to 109, wherein step (c) comprises modifying a polynucleotide encoding the parent variable domain to form a polynucleotide encoding a variable domain with a modified FR, wherein the modified FR has at least one amino acid position substituted with the amino acid in the corresponding position of the selected human subgroup variable domain consensus sequence.

110. The method of claim 109, wherein the polynucleotide further comprises a polynucleotide encoding a constant region domain connected to the polynucleotide encoding the variable domain with modified FR parent to form a polynucleotide encoding a full-length heavy or light chain.

111. The method of claim 110, wherein the polynucleotide further comprises an expression vector.

112. The method of claim 111, further comprising culturing a host cell comprising the expression vector; and recovering a full-length heavy or light chain or both from the culture.

113. The method according to claim 112, wherein the host cell is a prokaryotic host cell.

114. The method according to claim 112, wherein the host cell is a mammalian cell.

115. The method according to any of claims 104-115, wherein the variable domain is a heavy chain variable domain or a light chain variable domain.

116. The method according to claim 115, wherein the HVR1 amino acid sequence is GYTFTNYGIN (SEQ ID NO: 14), GYDFTHYGMN (SEQ ID NO:18), or GYSITSGYSWN (SEQ ID NO:19) .

117. The method according to any of claims 104-116, wherein the framework region is selected from the group consisting of FR1, FR2, FR3, FR4, and mixtures thereof.

118. The method according to claim 117, wherein the human subgroup FR consensus sequence is a heavy chain FR1 sequence with a sequence selected from the group consisting of SEQ. ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3.

119. The method according to any of claims 104-118, wherein the yield of the antibody or antigen binding fragment with the modified FR is improved at least 2 fold compared to the parent antibody or antigen binding fragment.

120. The method according to claim 119, wherein the yield of the antibody or antigen binding fragment with the modified FR is improved at least 2 fold to 16 fold compared to the parent antibody or antigen binding fragment.

121. The method of any of claims 104-121, wherein at least two identified amino acid positions in at least one FR are substituted with amino acids in the corresponding position of the selected subgroup consensus sequence.

122. The method of claim 121, wherein the FR is a heavy chain FR1 and one of the identified amino acid positions is position 6 or position 23 or both, and the other position is selected from the group consisting of position 1, 11, 13, 18, 19, and mixtures thereof.

123. The method of claim 122 wherein amino acid positions 6 and 23 are substituted.

124. The method of claim 122, wherein all of the amino acid positions at position, 1,6,11,13,18,19, and 23 of the heavy chain FR1 are substituted.

125. The method of any of claims 104-124, wherein all of the identified amino acid positions in a FR are each substituted with the amino acid in the corresponding position in the selected subgroup consensus sequence.

126. The method of claim 125, wherein the FR is FR1, FR2, or FR3.

127. The method of any of claims 104-126, wherein all of the identified amino acid positions in all FR are each substituted with the amino acid in the corresponding position in the selected subgroup consensus sequence.

128. A variable domain of an antibody or antigen binding fragment produced by the method of any of claims 1-74 and 82-126.

Fig. 1A

Fig. 1B

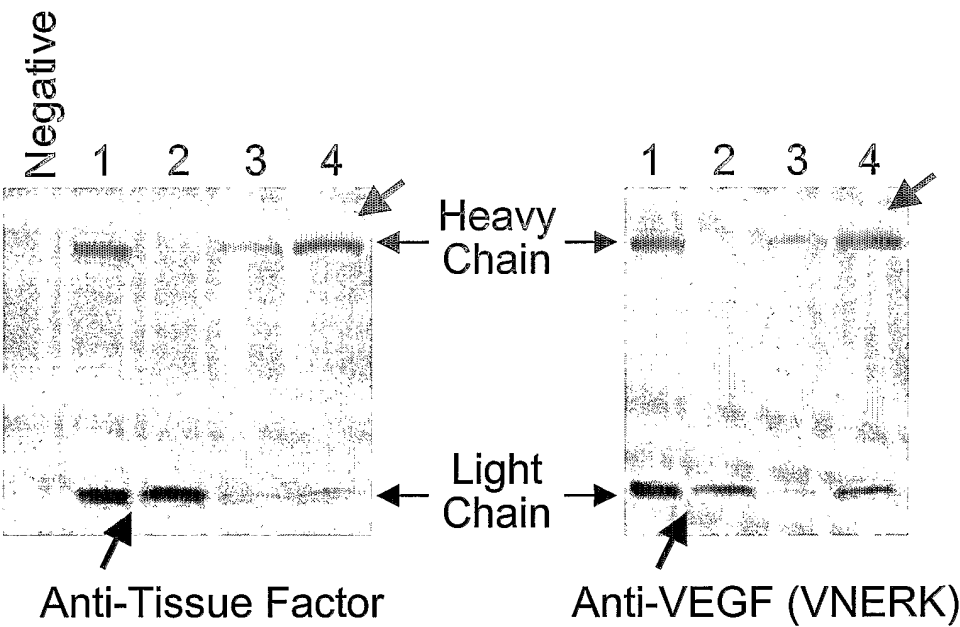


Fig. 2

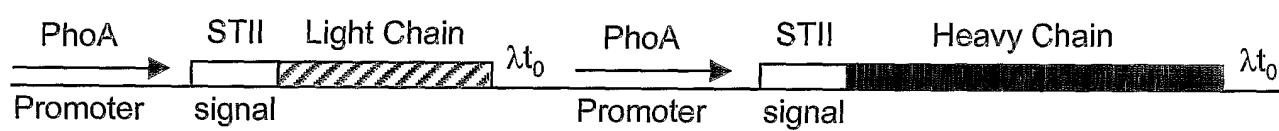


Fig. 3A

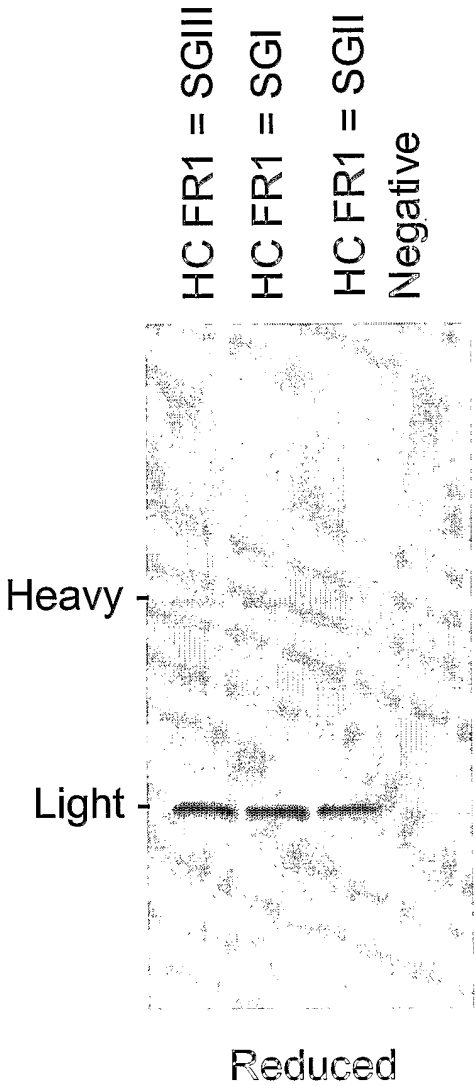


Fig. 3B

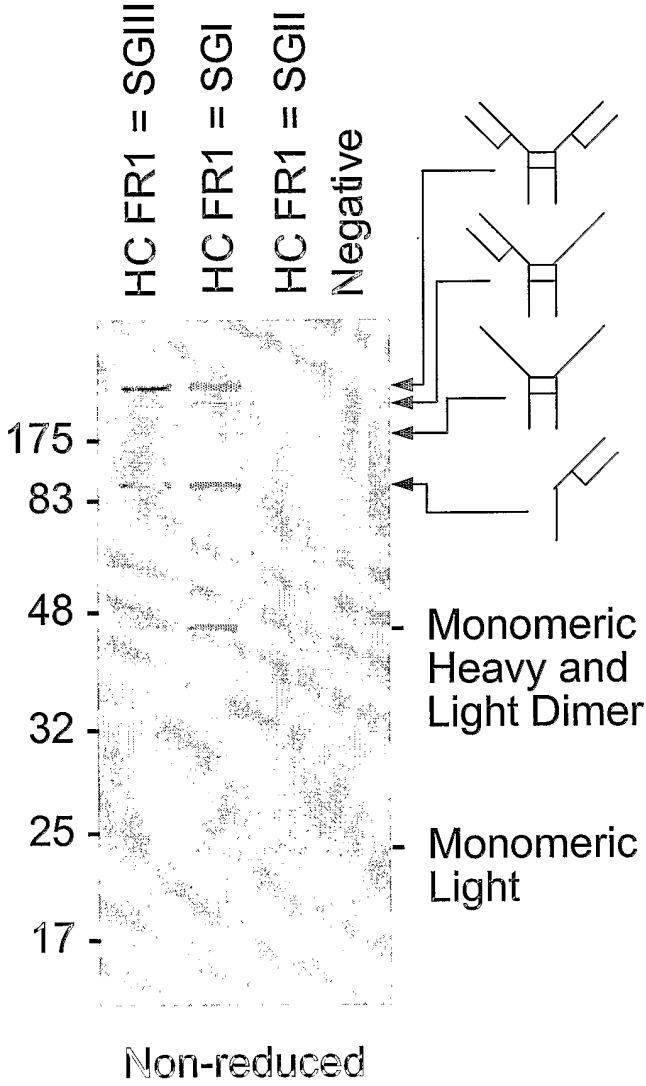


Fig. 4A

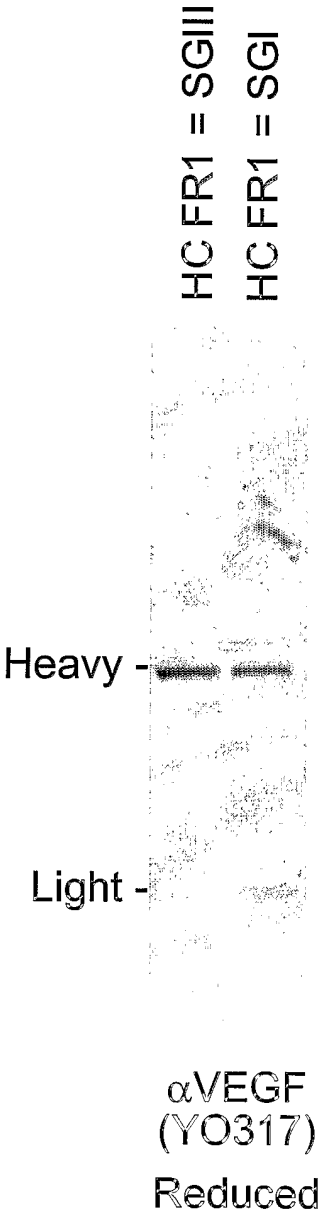


Fig. 4B

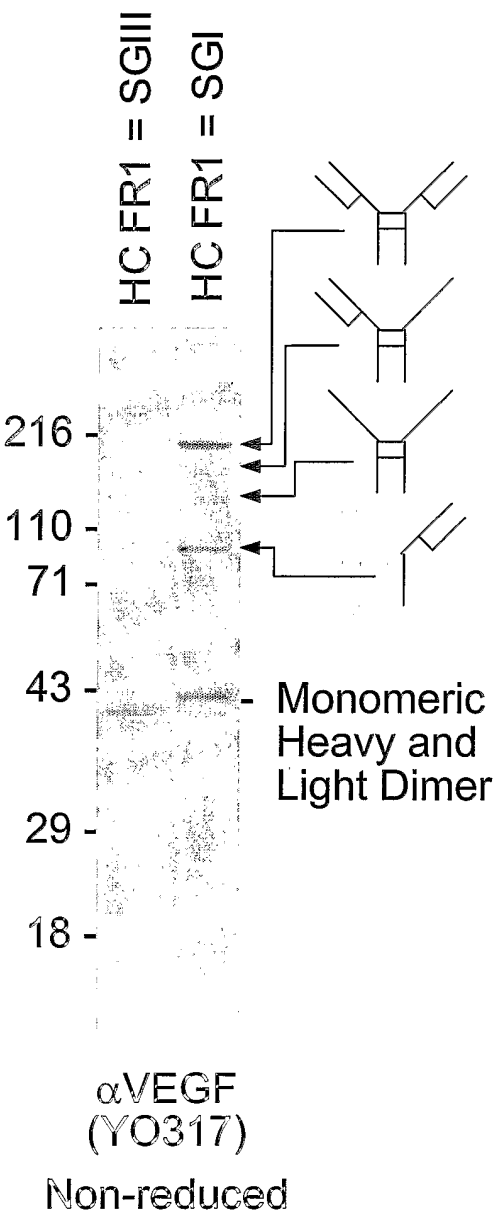


Fig. 5A

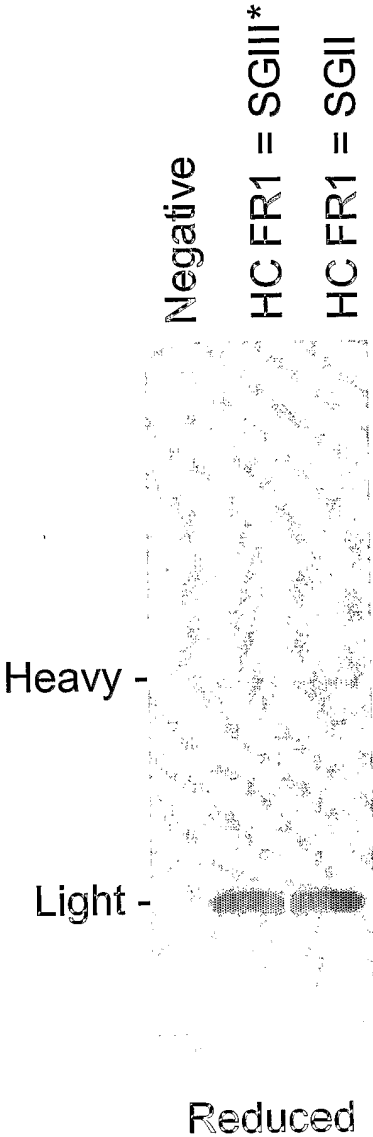
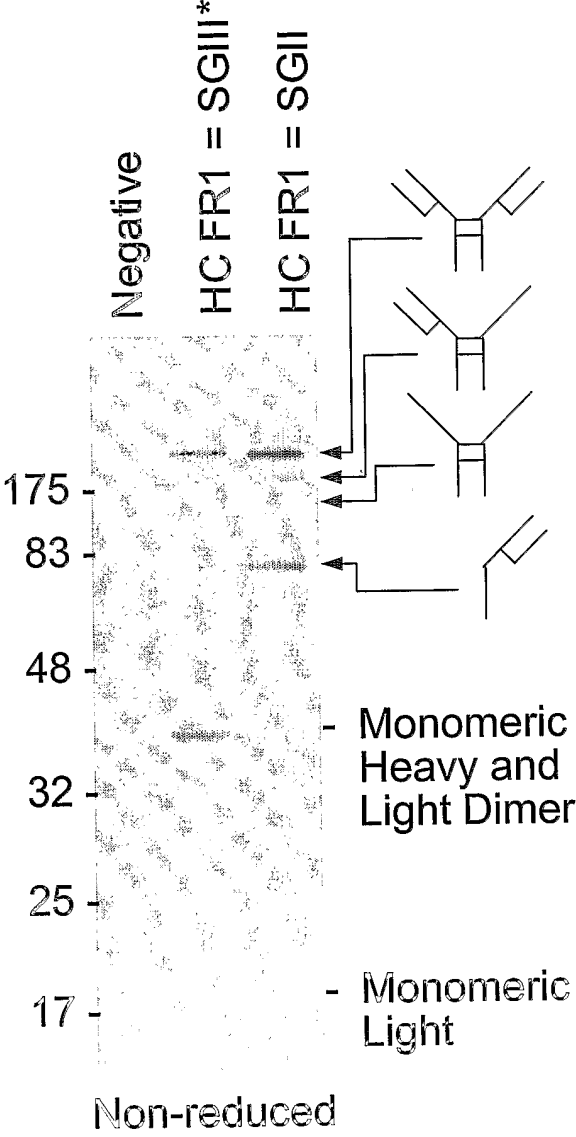


Fig. 5B



*Includes an A24V change as part of humanization.

Fig. 6A



Fig. 6B

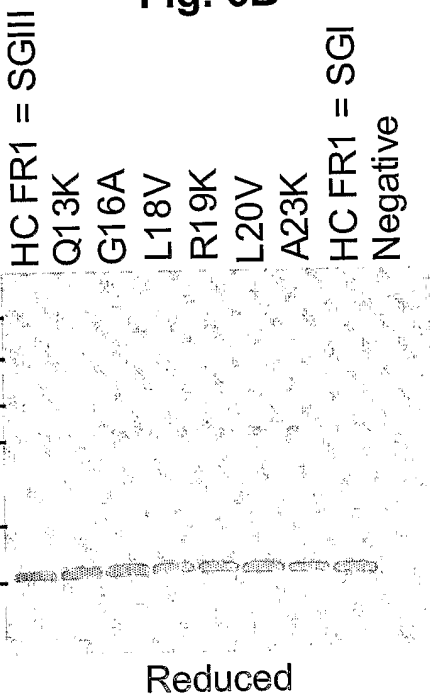


Fig. 6C

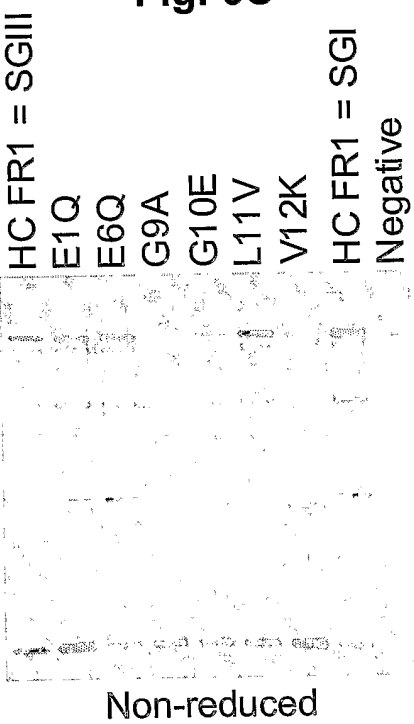


Fig. 6D

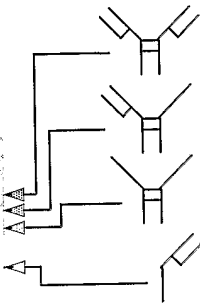
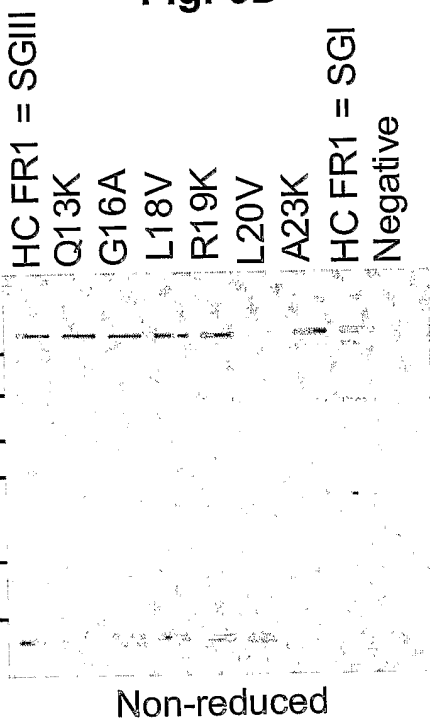


Fig. 7A

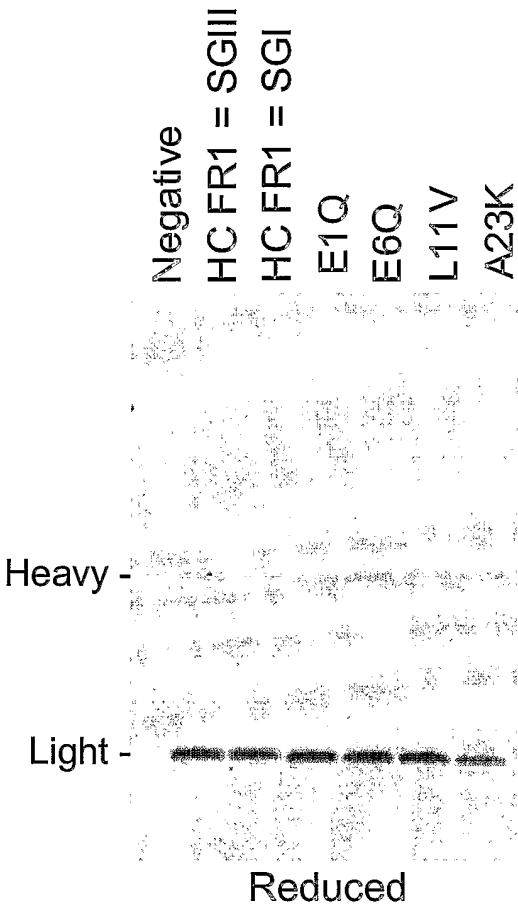


Fig. 7B

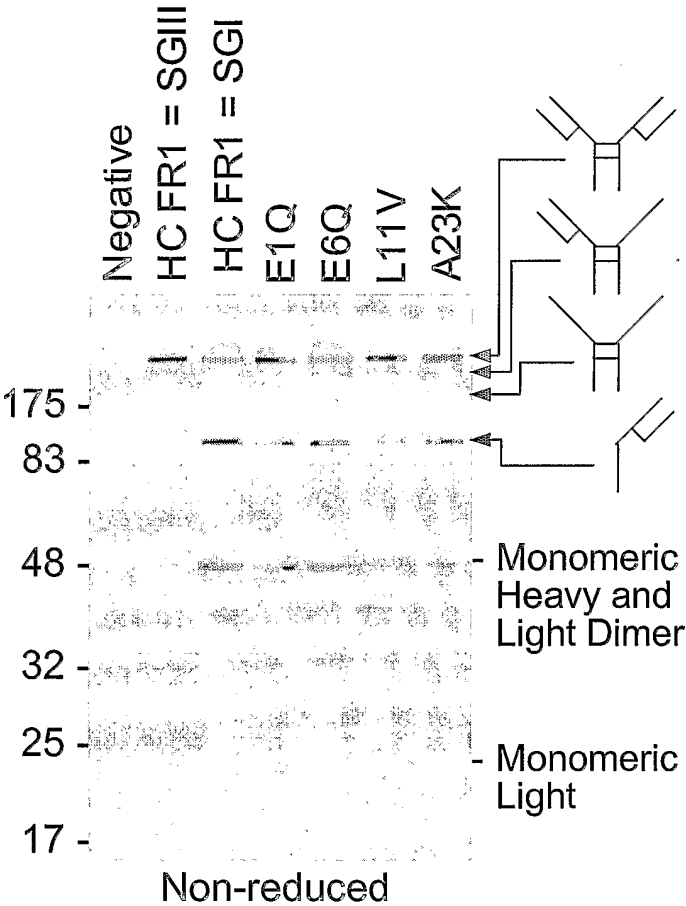


Fig. 8A

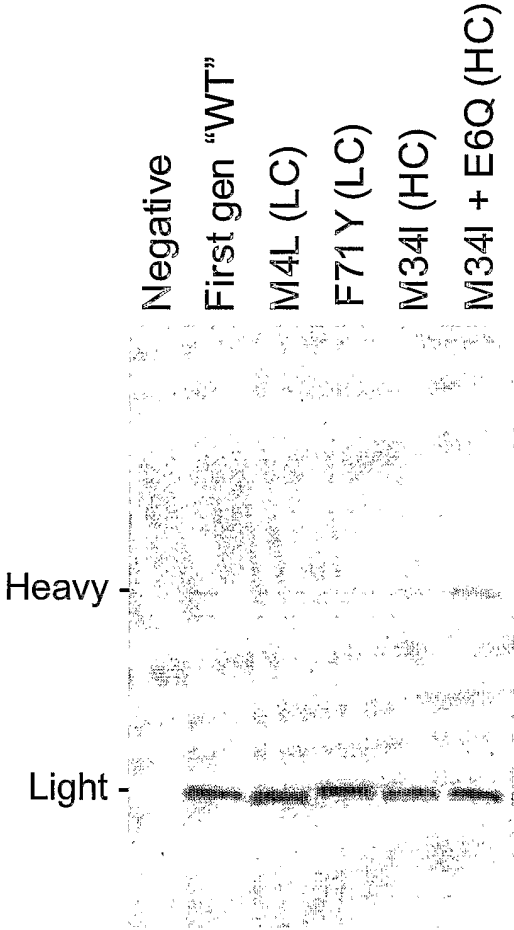


Fig. 8B

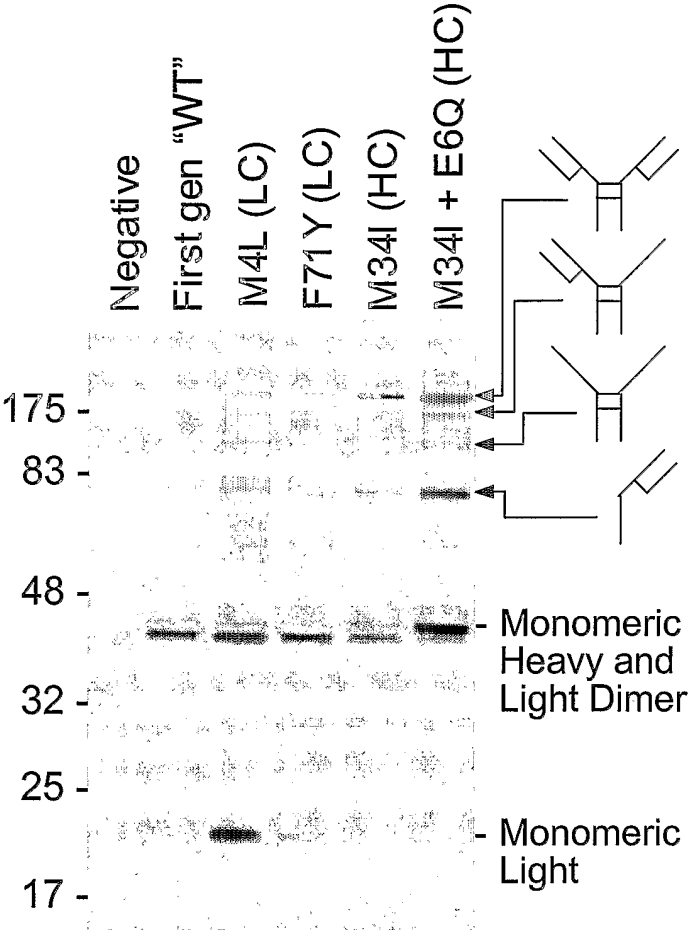


Fig. 9A

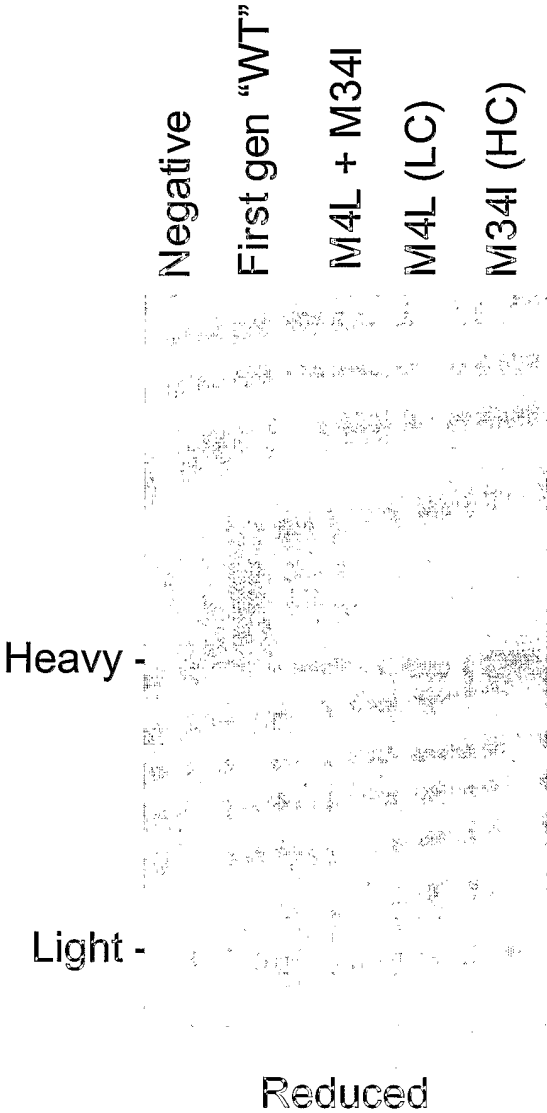


Fig. 9B

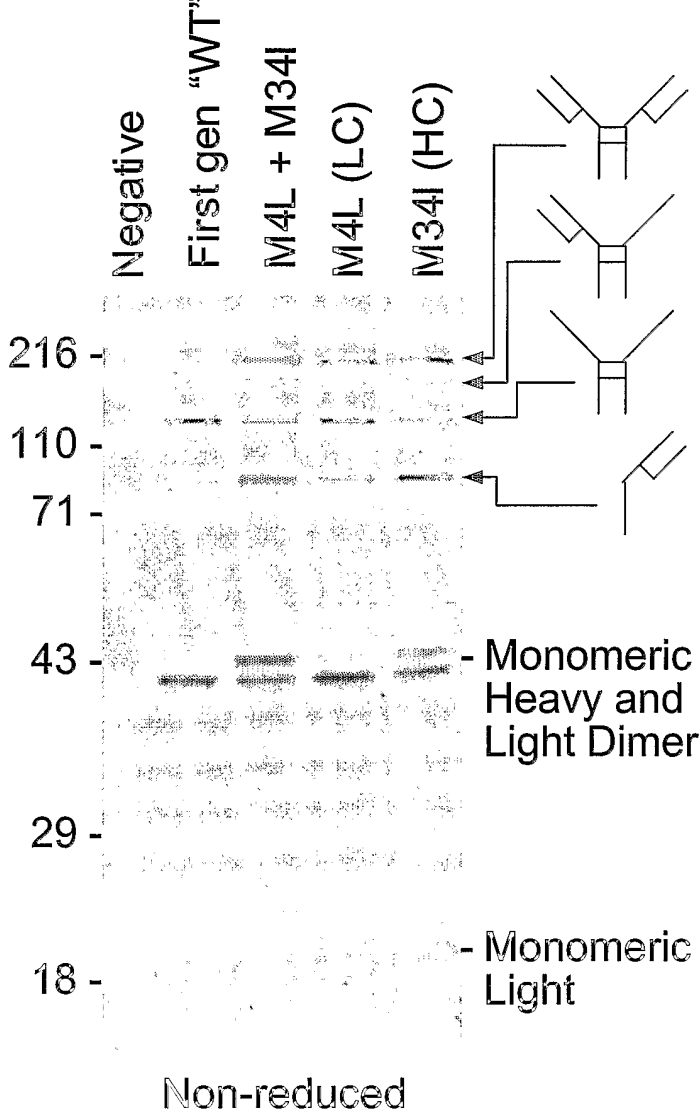


Fig. 10A

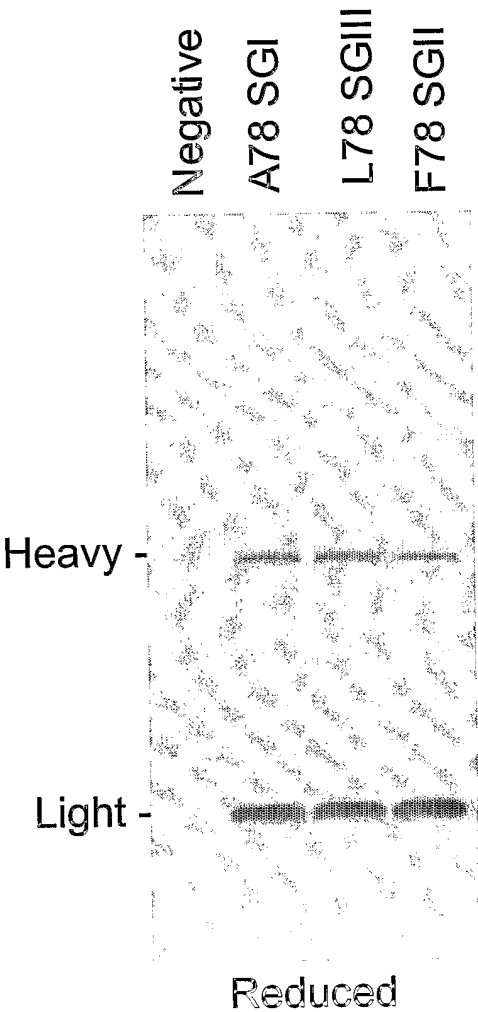


Fig. 10B

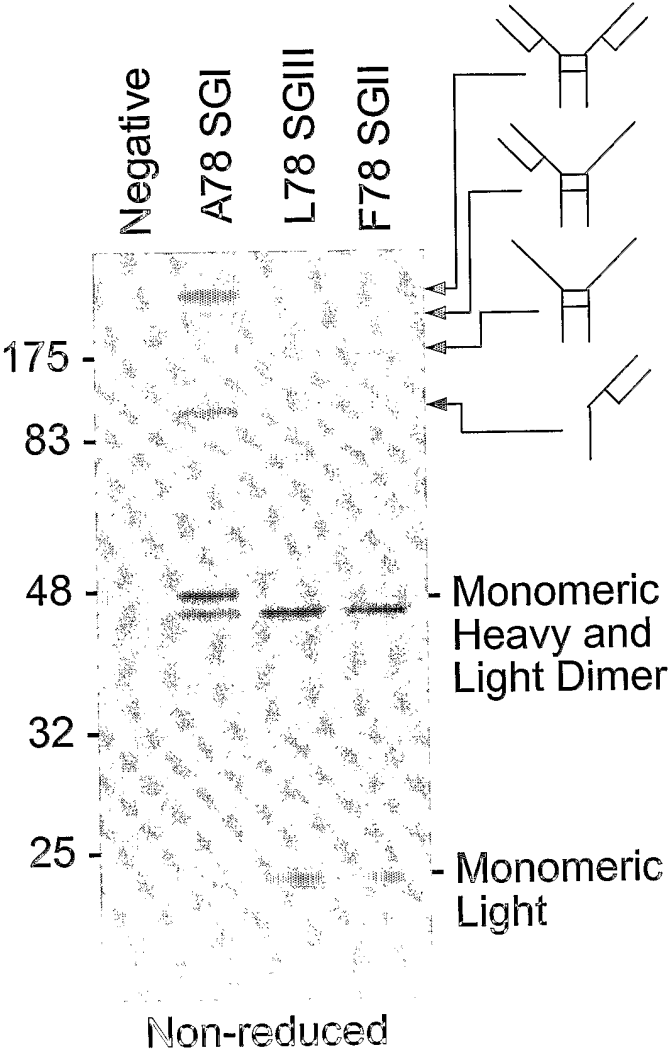


Fig. 11A

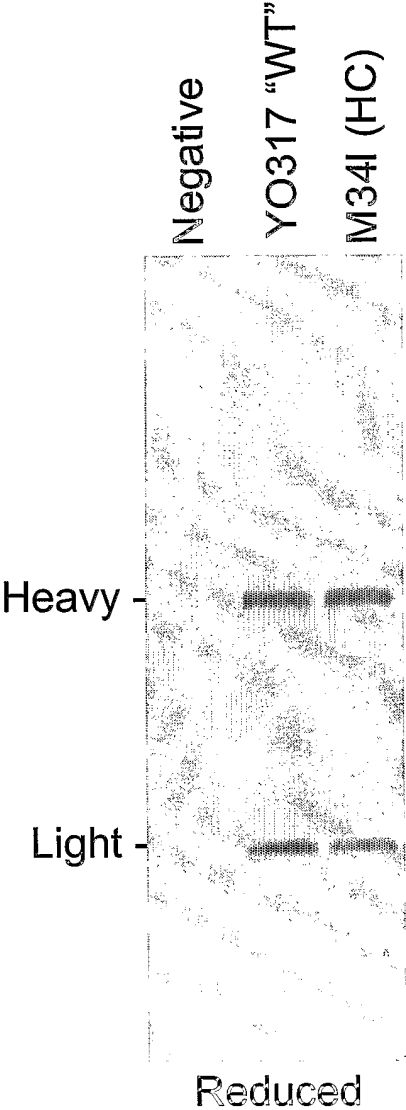


Fig. 11B

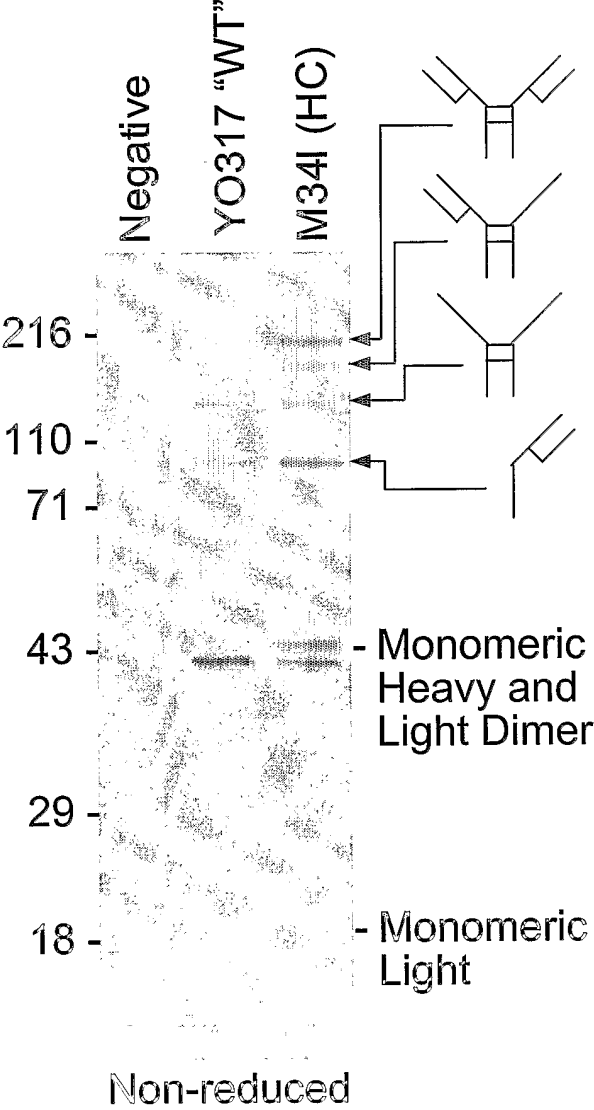


Fig. 12A

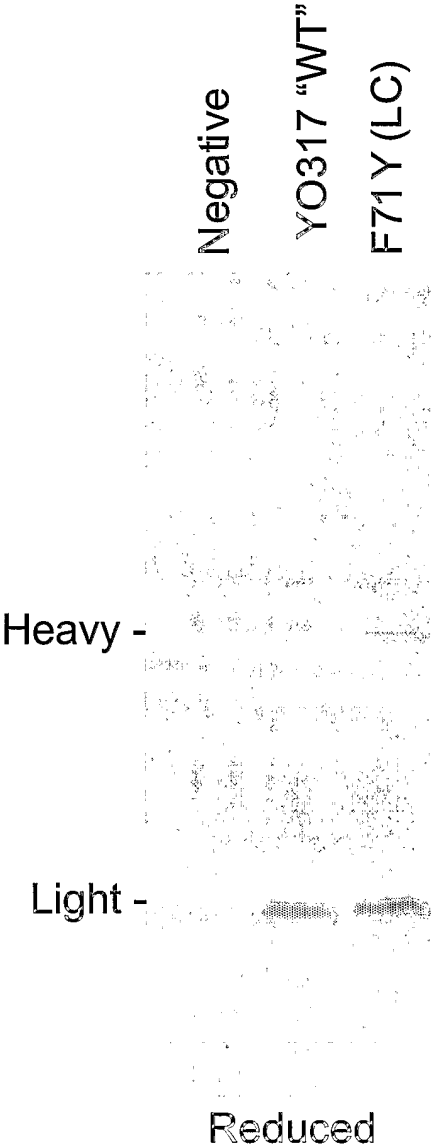


Fig. 12B

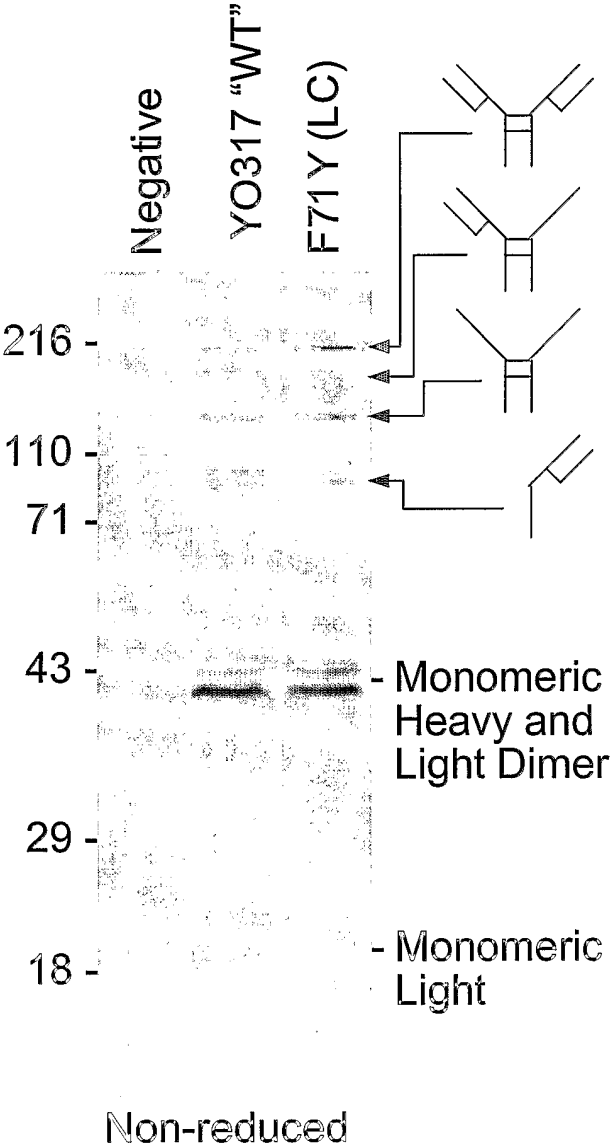


Fig. 13A

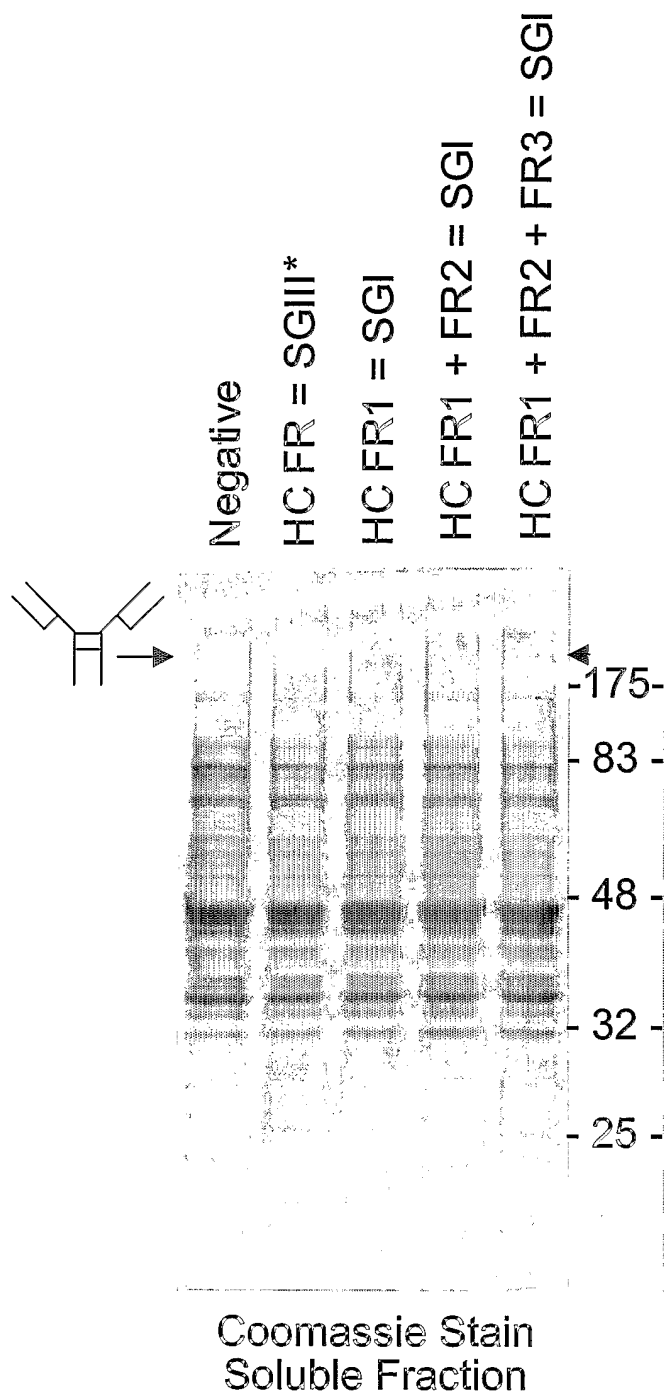
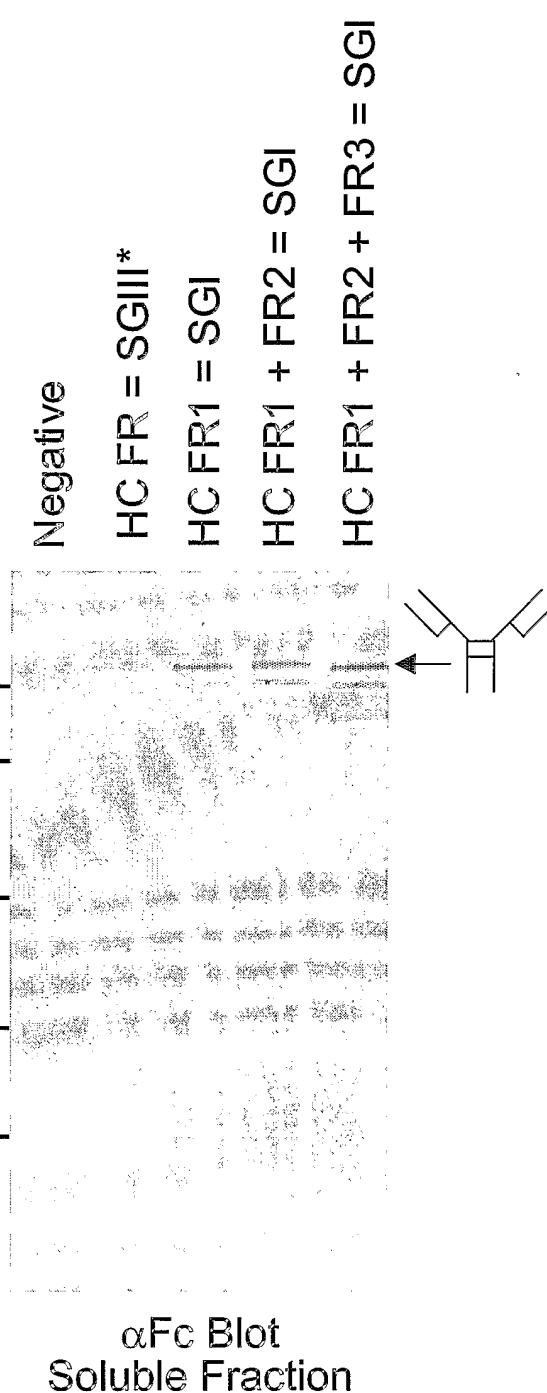


Fig. 13B



* Except for residues changed during humanization.

Fig. 14A

Fig. 14B

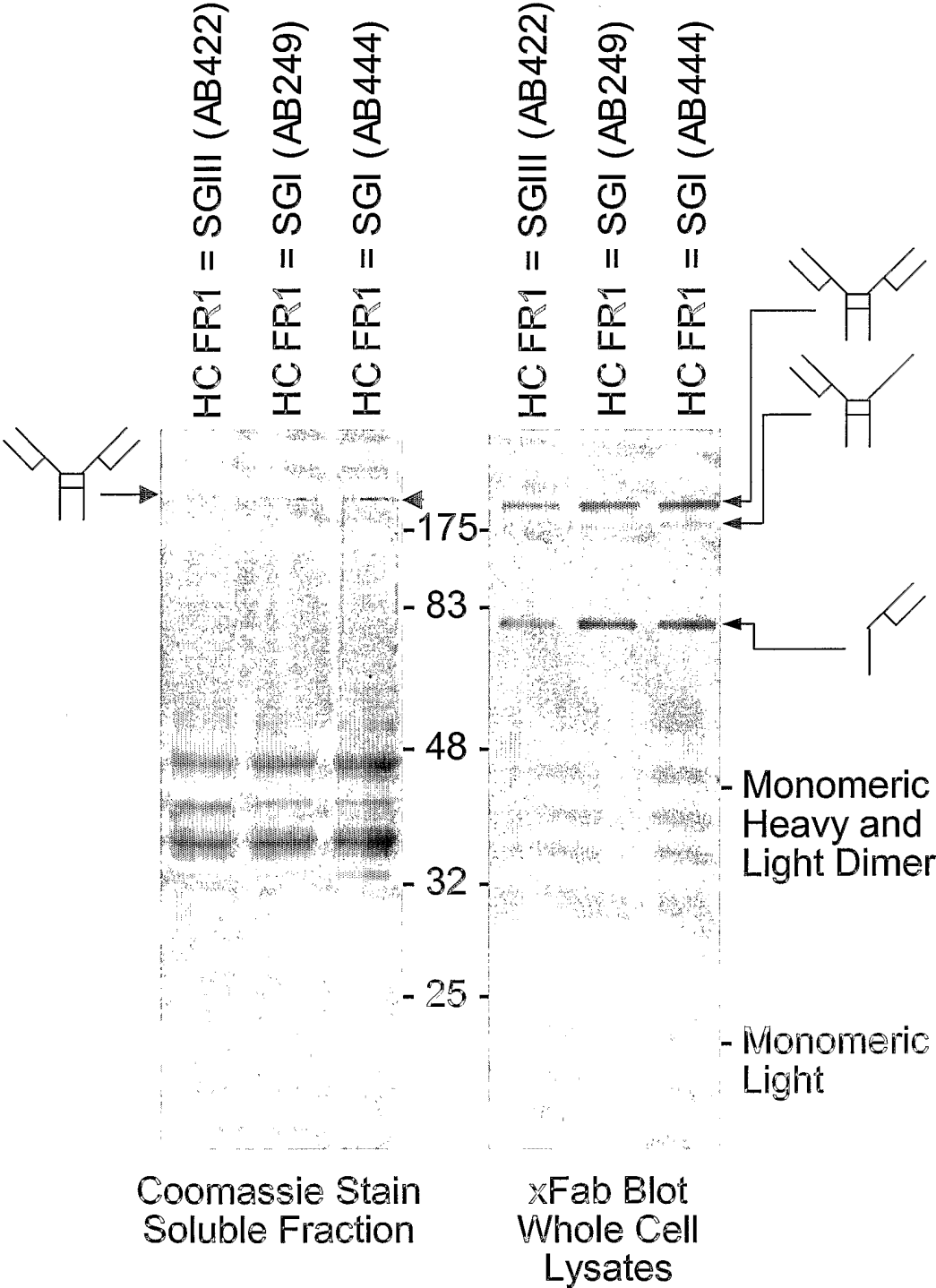


FIG. 15A

```

1  GAATTCACCT TCTCCATACT TTGATTAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA GTTGTATTAT AAGCTTGCCC AAAAAGAAGA AGAGTCGAAT
   CTTAAGTTGA AGAGGTATGA AACCTATTCC TTTATGTCTG TACTTTTAG AGTAACGACT CAACAATAAA TTCGAACGGG TTTTCTTCT TCTCAGCTTA

101  GAACTGTGTG CGCAGGTAGA AGCTTTGGAG ATTATCGTCA CTGCAATGCT TCGCAATATG GCGCAAAATG ACCAACAGCG GTTGATTGAT CAGGTAGAGG
   CTTGACACAC GCGTCCATCT TCGAAACCTC TAATAGCAGT GACGTTACGA AGCGTTATAC CCGGTTTTAC TGGTTGTGCG CAACATACTA GTCCATCTCC

201  GGGCGCTGTA CGAGGTAAAG CCCGATGCCA GCATTCTCTGA CGAGATACG GAGCTGCTGC GCGATTACGT AAAGAGTTA TTGAAGCATC CTCGTCAGTA
   CCCGCGACAT GCTCCATTTT GGGCTAGGGT CGTAAGGACT GCTGCTATGC CTGACGAGCG CGCTAATGCA TTTCTTCAAT AACTTCGTAG GAGCAGTCAT

301  AAAAGTTAAT CTTTTCACCA GCTGTCTATA AGTTGTCACG GCCGAGACTT ATAGTCGCTT TGTTTTATTT TTTAATGTA TTGTAACTA GTACGCAAGT
   TTTTCAATTA GAAAAGTTGT CGACAGTATT TCAACAGTGC CGGCTCTGAA TATCAGCGAA ACAAAAATAA AAAATTACAT AAACATTGAT CATGCGTTCA

401  TCACGTAAA AGGTAATCTA GAAATTATGAA GAAGATATC GCATTCTTTC TTGCATCTAT GTTCGTTTTT TCTATTGCTA CAAACCGGTA CGTCGATATC
   AGTGCATTTT TCCCATAGAT CTTAATACTT CTTCTTATAG CGTAAAGAAG AACTAGATA CAAGCAAAA AGATAACGAT GTTTGCGCAT GCGACTATAG
   M K K N I A F L L A S M F V F S I A T N A Y A D I
   ^STII Signal TIR -1
   ^variable light (VL) cys
   anti-VEGF Light Chain^

501  CAGTTGACCC AGTCCCGGAG CTCCTGTGTC GCCTCTGTGG GCGATAGGCT CACCATCACG TGCAGCGCAA GTCAGGATAT TAGCXAATAT TTAAACTGGT
   GTCAACTGGG TCAGGGGCTC GAGGGACAGG CCGACACACC CGCTATCCCA GTGCTAGTGG ACCTCGCGTT CAGTCCCTATA ATCGTTGATA AATTGACCA

26 Q L T Q S P S S L S A S V G D R V T I T C S A S Q D I S N Y L N W Y
   ^variable light (VL) cys

601  ATCAACAGA ACCAGGAAA GCTCCGAAAG TACTGATTTA CTTACCTCC TCCTCTCCACT CTGGAGTCCC TTCTCGCTTC TCTGGATCCG GTTCTGGGAC
   TAGTTGTCTT TGGTCCCTTT CGAGGCTTTC ATGACTAAAT GAACTGGAGG AGAGAGGTGA GACCTCAGGG AAGAGCGAAG AGACCTAGGC CAAGACCCCTG

60 Q Q K P G K A P K V L I Y F T S S L H S G V P S R F S G S G S G T

701  GGATTTCACT CTGACCATCA GCACTCTECA GCCAGAAGAC TTCGCAACTT ATTACTGTCA ACAGTATAGC ACCGTGCGGT GGACGTTTGG ACAGGTATCC
   CCTAAAGTGA GACTGGTAGT CGTCACACGT CCGTCTTCTG AAGCGTTGAA TAATGACAGT TGTATATCG TGGCACGGCA CCGTCAAAAC TGTCCTATGG

93 D F T L T I S S L Q P E D F A T Y C Q Q Y S T V P W T F G Q G T
   ^Variable light cys

801  AAGTGGAGA TCAAAACGAAC TGTGGCTGCA CCATCTGTCT TCACTCTCCC GCCATCTGAT GAGCAGTTGA AATCTGGAAC TGCTTCTGTT GTGTGCTTGC
   TTCCACCTCT AGTTGTCTTG ACACCGACGT GGTAGACAGA AGTAAAGGG CGGTAGACTA CTGCTCAACT TTAGACCTTG ACGAAGACAA CACACGGACG

126 K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L L
   Constant Light cys^

```

FIG. 15B

901 TGAATAACTT CTATCCAGA GAGGCCAAAG TACAGTGAA GGTGATAAC GCCTCCAAAT CCGGTAACCT CCAGAGAGT GTCACAGAGC AGACAGCAA
 ACTTATTGAA GATAGGCTCT CTCGGTTTC ATGTACACCTT CCACCTATTG CCGGAGGTTA GCCCATTCAG GGTCTCTCA CAGTGTCTCG TCCTGTCTGT
 160 N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K
 1001 GGACAGACCC TACAGCCTCA GCAGCACCTT GAGGTGAGC AAGCAGACT ACAGAAACA CAAAGTCTAC GCCTGCGAAG TCACCCATCA GGGCCTGAGC
 CCTGTCTGTG ATGTCCGAGT GTCGTGGGA CTGCGACTCG TTTCTCTCTGA TGTCTTTTGT GTTTCAGATG CGGACGCTTC AGTGGGTAGT CCCGACTCG
 193 D S T Y S L S S₁ T L T L S K A D Y E K H K V Y A C E V T H Q G L S
 ^Constant Light cys
 1101 TCGCCCGTCA CAAAGAGCTT CAACAGGGGA GAGTGTAAAT TAAATCTCTT ACGCCGGAGG CATCGTGGG AGCTCGGTAC CCGGGGATCT AGGCCTAACG
 AGCGGGCAGT GTTCTCGAA GTTGTCCTCT CTCACAATTA ATTTAGGAGA TGGCGGCTGC GTAGCACCGC TCGAGCCATG GGGCCCTAGA TCGGATTGC
 226 S P V T K S F N R G E C O
 ^cys to bind heavy start lambda t0 terminator^
 1201 CTCGGTTGCC GCGGGCGCTT TTTTATGTT GCCGAGCGC ATCTCGAATG AACTGTGTG CGAGGTAGAA GCTTTGGAGA TTATCGTCAC TGCAATGCTT
 GAGCCAACGG CGGCCCGCAA AAAATAACAA CCGCTGCGG TAGAGCTTAC TTGACACACG CPTCCATCTT CGAAACCTCT AATAGCAGTG ACGTTACGAA
 ^end lambda t0 terminator
 1301 CGCAATATGG CGCAAAATGA CCAACAGCGG TTGATGTATC AGGTAGAGGG GGGCGTGTAC GAGGTAAAG CCGATGCCAC CATTCCTGAC GACGATACGG
 GCGTTATACC GCGTTTACT GGTGTGCGC AACTAACTAG TCCATCTCCC CCGGACATG CTCCATTTCG GGTACGGTC GTAAGGACTG CTGCTATGCC
 1401 AGCTGTCTGG CGATTACGTA AAGAAGTTAT TGAAGCATCC TCGTCAGTAA AAGTTAATC TTTTCAACAG CTGTCAATAA GTTGTCACGG CCGAGACTTA
 TCAGCAGCGC GCTAATGCAT TTCITCAANA ACTTCGTAGG AGCAGTCATT TTTCAATTAG AAAAGTTGTC GACAGTATTT CAACAGTGCC GGCTCTGAAT
 1501 TACGCGCTTT GTTTTATTT TTTAATGTAT TTGTAACCTAG TACGCAAGTT CACGTAAAAA GGGTATCTAG AATTATGAAG AAGAATATCG CATTTCTTCT
 ATCAGCGAAA CAAAATAAAA AAATTACATA AACATGATC ATGCGTTCAA GTGCATTTTT CCCATAGATC TTAATACTTC TTCTTATAGC GTAAAGAAGA
 M K K N I A F L L
 ^STII signal TIR-1
 1601 TGCATCTATG TTGTTTITTT CTATGTCTAC AAACGGGTAC GCTGAGTTTC AGCTGGGTGA GTCTGGCGGT GGCCTGGTGC AGCCAGGGGG CTCACTCCGT
 ACGTAGATAC AAGCAAAA GATACGATG TTTGGGCATG CGACTCCAAG TCAGCCACTT CAGACCGCCA CCGGACCACG TCGGTCCCCC GAGTGAGGCA
 10 A S M F V F S I A T N A Y A E V Q L V E S G G L V Q P G G S L R
 ^anti-VEGF heavy chain (VNERK version)
 1701 TTGTCTCTTG CAGCTTCTGG CTATACCTTC ACCAACTATG GTATAAACTG GGTCCGTCAG GCGCCGGGTA AGGGCTCTGA ATGGTTTGA TGAATAACA
 AAACAGACAC GTCGAAGACC GATATGGAAG TGTTTGATAC CATATTTGAC CCAGGCAGTC CCGGGGCCAT TCCCGGACCT TACCCAACTT ACCTAATTGT
 43 L S C A A S G Y T F T N Y G I N W V R Q A P G K G L E W V G W I N T
 ^Variable Heavy (VH) cys
 1801 CCTATACGG TGAACCGACC TATGCTGCGG ATTTCAAACG TCGTTTCACT TTTTCTTTAG ACACCTCCAA AAGCACAGCA TACCTTCAGA TGAACAGCCT
 GGATATGCC ACTTGGCTGG ATACGACGCC TAAAGTTGC AGCAAGTGA AAAAGAAATC TGTGGAGTT TTCGTGTCGT ATGGAGTCT ACTTGTGCGA
 77 Y T G E P T Y A A D F K R R F T F S L D T S K S T A Y L Q M N S L

FIG. 15C

1901 GCGGCGCTGAG GAACTGCGG TCTATTACTG TGCAAGTAC CCGCACTATT ATGTGAACGA GCGGAAGAGC CACTGTGTATT TCGACGTCTG GGTCAAGGA
 CGCGGACTC CTGTGACGC AGATAATGAC AGTTTTCATG GCGGTGATAA TACACTTGCT CGCTTCTCG GTACCATAA AGCTGCAGAC CCCAGTTCCT
 110 R A E D T A V Y Y C A K Y P H Y Y V N E R K S H W Y F D V W G Q G
 ^VH cys
 2001 ACCCTGGTCA CCGTCTCCTC GGCCTCCACC AAGGGCCCAT CGGTCTTCCC CTTGGACCC TCCCTCCAAGA GCACCTCTGG GGGCACAGCG GCCCTGGGCT
 TGGGACCACT GGCAGAGGAG CCGGAGGTGG TTCCCGGGTA GCCAGAAGGG GGACCGTGGG AGGAGTTCT CGTGGAGACC CCCGTGTGCG CGGGACCCGA
 143 T L V T V S S A S T K G P S V F P L A P S S K S T S G G T A A L G C
 Constant Heavy 1 (CH1) cys^
 2101 GCCTTGCTCAA GGACTACTTC CCGAACCAGG TGACGGTGTG GTGGAATCA GGGGCCCTGA CCAGCGCGGT GCACACCTTC CCGGCTGTCC TACAGTCCTC
 CGGACCACTT CCTGATGAG GGGCTTGGCC ACTGCCACAG CACCTTGAGT CCGCGGGAAT GGTGCGCGCA CGTGTGGAAG GCGCGACAGG ATGTCAGGAG
 177 L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S
 2201 AGGACTCTAC TCCCTCAGCA GCGTGGTGAC TGTGCCCTCT AGCAGCTTGG GCACCAGAC CTACATCTGC AACGTGAATC ACAAGCCAG CAACACCAAG
 TCCTGAGATG AGGAGTCGT CGCACCACTG ACACGGGAGA TCGTCGAACC CGTGGTCTG GATGTAGACG TTGCACCTAG TGTTCGGGTC GTTGTGGTTC
 210 G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K
 ^CH1 cys
 2301 GTGGACAAGA AAGTTGAGCC CAATCTTGT GACAAACTC ACATATGCC ACCGTGCCA GCACCTGAAC TCCTGGGGG ACCGTAGTC TTCCTCTTCC
 CACCTGTTCT TTCAACTCG GTTAGAACA CTTCTTTGAG TGTGTACGG TGGACGGGT CGTGGACTTG AGGACCCCC TGGCAGTCAAG AAGGAGAAGG
 243 V D K K V E P K S C D K T H T C P P C P A P E L L G G P S V F L F P
 ^cys to bind light chain ^hinge cys
 2401 CCCCAAAACC CAAGACACC CTCATGATCT CCCGGACCCC TGAGGTCACTA TCGGTGTGAG TGGACGTGAG CCACGAAGAC CCTGAGGTCA AGTTCAACTG
 GGGGTTTGG GTTCCCTGTG GAGTACTAGA GGGGCTGGGG ACTCCAGTGT AGCACCACC ACTGCACATC GTGTCTCTG GGACTCCAGT TCAAGTTGAC
 277 P K P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W
 ^Constant Heavy 2 (CH2) cys
 2501 GPACGTGGAC GCGGTGGAGG TGCATAATGC CAACACAAG CCGCGGGAGG AGCAGTACAA CAGCAAGTAC CGTGTGGTCA GGTCTCTCAC CGTCTGTCAC
 CATGCACCTG CCGCACCTCC ACGTATTACG GTTCTGTGTTG GCGGCCCTCC TCGTCAATGT GTCTGTGATG GCACACCACT CGCAGGAGTG GCAGGACCTG
 310 Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H
 2601 CAGGACTGGC TGAATGGCAA GAGTACAAAG TGCAAGGTCT CCACAAAGC CTTCCAGCC CCCATCAGA AAACCATCTC CAAGGCCAAA GGGCAGCCCC
 GTCTGACCG ACTTACCGTT CCTCAATGTT CAGTTCCAGA GGTGTGTTG GAGGGTCCG GGGTAGCTCT TTGTGTAGAG GTTTCGTTT CCCGTCGGG
 343 Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R
 ^CH2 cys
 2701 GAGAACCA GGTGTACACC CTGCCCCCAT CCCGGAAGA GATGACCAAG AACAGGTCA GCCTGACCTG CCTGGTCAA GGTCTTCTATC CCAGCGACAT
 CTCTGTGTGT CCACATGTGG GACGGGGTA GGGCCCTTCT CTACTGGTTC TTGTGCCAGT CCGACTGGAC GGACCACTTT CCGAAGATAG GGTGCTGTGA
 377 E P Q V Y T L P P S R E E M T K N Q V S L T C L V K G F Y P S D I
 ^Constant Heavy 3 (CH3) cys

FIG. 15D

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2801 CGCGTGGAG TGGAGAGCA ATGGGAGCC GGAGAACAAAC TACAAGACCA CGCCTCCCGT GCTGGACTCC GACGGCTCCT TCTTCCTCTA CAGCAAGCTC
CGGGACCTC ACCCTCTCGT TACCCGTCGG CCTCTTGTTG ATGTTCTGGT GCGGAGGGCA CGACCTGAGG CTGCCGAGGA AGAAGGAGAT GTCGTTTCGAG
410 A V E W E S N G Q P E N N Y K T T P P V L D S D G S F F L Y S K L

2901 ACCGTGGACA AGAGCAGGTG GCAGCAGGGG AACGTCTTCT CATGCTCCGT GATGCATGAG GCTCTGCACA ACCACTACAC GCAGAAAGAGC CTCTCCCTGT
TGGCACCTGT TCTCGTCCAC CCTCGTCCC CTGCAAGA GTACGAGGCA CTACGTACTC CGAGACGTTG TGGTGAATG CGTCTTCTCG GAGAGGGACA
443 T V D K S R W Q , Q G N V F S C S V M H E A L H N H Y T Q K S L S L S
^CH3 cys

3001 CTCGGGGTAA ATAAGCATGC GACGGCCCTA GAGTCCCTAA CGCTCGGTTG CCGCGGGCGG TTTTATTG TTAACCTCATG JTTGACAGCT TATCATCGAT
GAGGCCCAT TATTGTCAG CTGCGGGAT CTCAGGGATT GCGAGCCAAC GCGGCCCGC AAAAAATAAC AATTGAGTAC AAACCTGTCA ATAGTAGCTA
477 P G K O
^start lambda t0 terminator ^end lambda t0 terminator
^start of tet resistance promoter ^start of tet resistance promoter
^-35 of promoter

3101 AAGCTTTAAT GCGGTAGTTT ATCACAGTTA AATTGCTAAC GCAGTCAGG ACCGTGTATG AAATCTAACA ATGCGCTCAT CGTCATCCTC GGCACCGTCA
TTCGAAATTA CGCATCAAA TAGTGTCAT TTAACGATTG CGTCAGTCCG TGGCACATAC TTTAGATTGT TACGCGAGTA GCAGTAGGAG CCGTGGCAGT
^-10 region of tet resistance promoter ^start of tet resistance translation

3201 CCTTGATGC TGTAGGCATA GGTGTGTTA TGCCGGTACT GCCGGCCTC TTGCGGGATA TCGTCCATTG CGACAGCATC GCCAGTCAGT ATGGCGTGT
GGGACCTACG ACATCCGTAT CGAACCAAT ACGGCCATGA CCGCCCGGAG AACGCCCTAT AGCAGGTTAG GCTGTCCGTAG CCGTCAGTGA TACCGCACGA
3301

```

FIG. 16A

1 GAATTTCAACT TCTCCATACT TTGGATAAAG AAATACAGAC ATGAAAAATC TCAATGCTGA GTTGTATTAT AAGCTTGCCC AAAAAAGA AGAGTCGAAT
 CTTAAGTTGA AGAGGTATGA AACCTATTCC TTATGCTCTG TACTTTTAG AGTAACGACT CAACAATAAA TTTCGAACGGG TTTTTCCTCT TCTCAGCTTA
 101 GAATGTGTG CGCAGGTAGA AGCTTTGGAG ATTATGCTCA CTGCAATGCT TCGCAATATG GCGCAAAATG ACCAACAGCG GTTGATTGAT CAGGTAGAGG
 CTTGACACAC GCGTCCATCT TCGAAACCTC TAATAGCAGT GACGTACGA AGCTTATAC CCGGTTTTAC TGGTGTGCG CAACCTAATA GTCCATCTCC
 201 GGGCGCTGA CGAGGTAAAG CCCGATGCCA GCATTCCCTGA CGACGATACG GAGCTGTGTC GCGATTACGT AAAGAAGTTA TTGAAGCATC CTCGTCAGTA
 CCCCGACAT GCTCCATTTC GGGCTACGGT CGTAAGGACT GCTGCTATGC CTCGACGACG CGCTAATGCA TTCTTTCAAT AACTTCGTAG GAGCAGTCAT
 301 AAAAGTTAAT CTTTTCACCA GCTGTCAATA AGTTGTCAAG GCCGAGACTT ATAGTCGCTT TGTTTTATT TTTTAAATGA TTGTAACTA GTACGCAAGT
 TTTTCAATTA GAAAGTTGT CGACAGTATT TCAACAGTGC CGGCTCTGAA TATCAGCGAA ACAAAAATAA AAAATTACAT AAACATTGAT CATGCGTTCA
 401 TCAGGTAAA AGGTATCTA GAAATTATGA GAAGAATATC GCATTTCCTC TTGCATCTAT GTTCGTTTTT TCTATTGCTA CAAACGCGTA CGTGTATATC
 AGTGCATTTT TCCCATAGAT CTTAATACTT CTCTTATATAG CGTAAAGAAG AACGTAGATA CAAGCAAAA AGATAACGAT GTTTGCGCAT GCGACTATAG
 1 M K K N I A F L L A S M F V F S I A T N A Y A D I
 Anti-VEGF Light chain (version Y0317)^
 ^STII Signal TIR -1
 501 CAGTTGACCC AGTCCCGAG CTCCTGTGTC GCGATAGGT CACCATCACC TGCAGCGCAA GTACAGGATAT TAGCAACTAT TTAAACTGGT
 GTCAACTGG TCAGGGGCTC GAGGGACAGG CGGAGACACC CGCTATCCCA GTGTAGTGG ACCTCGCGTT CAGTCCCTATA ATCGTTGATA AATTGACCA
 26 Q L T Q S P S S L S A S V G D R V T I T C S A S Q D I S N Y L N W Y
 601 ATCAACAGAA ACCAGGAAA GCTCCGAAAG TACTGATTTA CTTACCTCC TCTCTCCACT CTGGAGTCCC TTCTCGCTTC TCTGATCCG GTTCTGGGAC
 TAGTTGTCTT TGGTCCCTTT CGAGGCTTTC ATGACTPAAAT GAAGTGGAGG AGAGAGGTGA GACCTCAGG AAGAGCGAAG AGACCTAGGC CAAGACCTG
 60 Q Q K P G K A P K V L I Y F T S S L H S G V P S R F S G S G S G T
 701 GGATTTTCACT CTGACCATCA GCAGTCTGCA GCCAGAGAC TTCCGCAACTT ATTACTGTCA ACAGTATAGC ACCGTGCCGT GGACGTTTGG ACAGGTTACC
 CCTAAAGTGA GACTGGTAGT CGTCAGACGT CCGTCTCTCTG AAGCGTTGAA TAATGACAGT TGTCTATATCG TGGCACCGCA CCTGCAACC TGTCCCATGG
 93 D F T L T I S S L Q P E D F A T Y Y C Q Q Y S T V P W T F G Q G T
 801 AAGTTGGAGA TCAAAACGAAC TGTGGCTGCA CCATCTGTCT TCATCTTCCC GCCATCTGAT GAGCAGTTGA AAFCTGGAAC TGCTTCTGTT GTGTGCTGTC
 TTCCACCTCT AGTTTGCTTG ACACCGACGT GGTAGACAGA AGTAAGAGG CGGTAGACTA CTGCTCACT TTAGACCTTG ACGAAGACAA CACACGACG
 126 K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L L L
 901 TGAATAACTT CTATCCAGA GAGGCCAAAG TACAGTGGAA GGTGATAAC GCCCTCCAAT CCGGTAACTC CCAGGAGAGT GTACACAGAGC AGGACAGCAA
 ACTTATTGAA GATAGGGTCT CTCGGGTTTC ATGTCACTT CCACCTATTG CCGGAGGTGA GCCCATGAG GTCTCTCTCA CAGTGTCTCG TCCTGTCTGTT
 160 N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K

FIG. 16B

1001 GGACAGCACC TACAGCCTCA GCAGCACCT GAGCTGAGC AAAGCAGACT ACAGAAACA CAAAGTCTAC GCTGCGAAG TCACCATCA GGGCCTGAGC
 CCTGTGCTGG ATGTCGGAGT CGTCGCTGGA TTTCGTCTGA TGCTCTTTGT GTTTCAGATG CGGACGCTTC AGTGGGTAGT CCGGACTCG
 193 D S T Y S L S S T L T L S K A D Y E K H K V Y A C E V T H Q G L S
 1101 TCGCCGCTCA CAAGAGCTT CAACAGGGA GAGTGTAAAT TAAATCTCT ACGCCGAGC CATCTGGCG AGCTCGGTAC CCGGGATCT AGGCCTAACG
 AGCGGCAGT GTTCTCGAA GTTGTCCCT CTCACAATTA ATTAGGAGA TCGGCTGTC GTAGCACCG TCGAGCCATG GGGCCCTAGA TCGGATTCG
 226 S P V T K S F M R G E C O
 1201 CTCGCTGCC GCCGGCGTT TTTTATTGTT GCCAGCGC ATCTCGAATG AACTGTGTC GCAGGTAGAA GCTTTGGAGA TTATCGTCAC TGCAATGCTT
 GAGCCAACGG CGGCCGCAA AAATAACAA CGGCTGCGG TAGAGCTTAC TTGACACACG CGTCCATCTT CGAACCTCT AATAGCAGTG ACGTTACGAA
 1301 CGCAATATGG CGCAAAATGA CCAACAGCG TTGATTGATC AGGTAGAGG GCGCTGTAC GAGGTAAAG CCGATGCCAG CATTCCTGAC GACGATACGG
 GCGTTATACC GCGTTTACT GGTGTCGCC AACTAACTAG TCCATCTCCC CCGCGACATG CTCCTTTTCG GGTACGGTTC GTAAGGACTG CTGCTATGCC
 1401 AGCTGTGTCG CGATTACGTA AAGAAGTTAT TGAAGCATCC TCCTCAGTAA AAGTTAATC TTTTCAACAG CTGTCAATAA GTTGTCAAG CCGAGACTTA
 TCGACGAGC GCTAATGCAT TTCTTCAATA ACTTCGTAGG AGCAGTCAAT TTTCAATTAG AAAAGTTGTC GACAGTATTT CAACAGTGCC GGCTCTGAAT
 1501 TAGTCGCTTT GTTTTATTT TTTAATGTAT TTGTAACCTAG TACGCAAGTT CACGTAAAA GGTATCTAG AATTATGAG AAGAATATCG CATTCTTCT
 ATCAGCGAAA CAAAAATAA AAATTACATA AACATGTATC ATGCGTTCAA GTGCATTTT CCATAGATC TTAATATTC TTTTATAGC GTAAGAAGA
 1601 TGCATCTATG TTCGTTTTTT CTATTGCTAC AAACGGTAC GCTCAGGTTT AGTGTGTGA GTCTGGCGT GGCCTGTGTC AGCCAGGGG CTCACTCCGT
 ACGTAGATAC AAGCAAAAA GATAACGATG TTTGGGCATG CGACTCCAG TCGACCACT CAGACCGCA CGGACCCAG TCGTCCCCC GAGTGAGGCA
 10 A S M F V F S I A T N A Y A E V Q L V E S G G L V Q P G G S L R
 ^Anti-VEGF Heavy Chain (version Y0317)
 1701 TTGRCCTGTG CAGCTTCTGG CTACGACTTC ACGCACTACG GTATGAAC TGTCGCTCAG GCCCGGGTA AGGGCCTGGA ATGGGTGGA TGGATTAAACA
 AACAGGACAC GTCGAAGACC GATGCTGAAG TGCGTGTATG CATACTGAC CAGGACATG CCAGGACAT CAGGACCACT CAGGACCACT TCCCGGACCT TACCCCAACCT ACCTAATTGT
 43 L S C A A S G Y D F T H Y G M N W V R Q A P G K G L E W V G W I N T
 1801 CCTATACCG TGAACCGACC TATGCTGCGG ATTCAAAACG TCGTTTCACT TTTTCTTTAG ACACCTCCAA AAGCACAGCA TACCTGACGA TGAACAGCCT
 GGATATGGC ACTTGGCTGG ATACGACGCC TAAAGTTGC AGCAATGTA AAAAGAAATC TGTGAGGTT TCGTGTGCT ATGACGCTCT ACTTGTGGA
 77 Y T G E P T Y A A D F K R R F T F S L D T S K S T A Y L Q M N S L
 1901 GCGGCTGAG GACACTGCCG TCTATTACTG TGCAAAGTAC CCGTACTATT ATGGACGAG CCACCTGGTAT TTCACGCTT GGGTCAAG AACCTTGGTC
 CGCGGACTC CTGTGACGCG AGATAATGAC ACGTTTTCATG GGCATGATAA TACCTGTCTC GGTGACATAA AAGTGCAGA CCCAGTTC TGGGACCAAG
 110 R A E D T A V Y Y C A K Y P Y Y Y G T S H W Y F D V W G Q G T L V
 2001 ACCGTCTCCT CGGCCTCCAC CAAGGGCCCA TCGGTCTTCC CCGTGGACC CTCCTCAAG AGCACTCTG GGGCACAGC GGCCTGGG TCCTGTGTC
 TGGCAGAGGA GCCGAGGTG GTTCCCGGT AGCCAGAGG GGGACCTGG GAGGAGTTT TCGTGGAGAC CCCGTGTG CCGGACCCG ACGGACCACT
 143 T V S S A S T K G P S V F P L A P S S K S T S G G T A A L G C L V K

FIG. 16C

2101 AGGACTACTT CCCCAACCG GTGACGGTGT CGTGAAGACT AGGCGCCCTG TGCACACCTT CCGGCTGTCT CTACAGTCCT CAGGACTCTA
 177 TCCGTGATGA GGGGCTTGGC CACTGCCACA GCACCTTAG TCCGCGGGAC TCGTCGGCGC ACCTGTGGAA GGGCCGACAG GATGTCAGGA GTCTTGAGAT
 177 D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S G L Y
 2201 CTCCTTCAGC AGCGTGTGA CTGTGCCCTC TAGCAGCTTG GGCACCCAGA CCTATCATCT CAAGTGAAT CACAAGCCCA GCACACCAA GTTGACACAG
 GAGGAGTGG TCGCACCACT GACACGGAG ATCGTCGAAC CCGTGGGTCT GATGTACAC GTTGCACTTA GTGTGCGGT CGTTGTGGTT CCACCTGTTC
 210 S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K
 2301 ABAATTGAGC CCAATCTTG TGACAAAAT CACATGCC CACGTGCC AGCACTGAA CTCTGGGG GACCTCACT CTTCCTCTTC CCCCCAAAAC
 TTTCAACTCG GGTITAGAAC ACTGTTTGA GTGTGTACGG GTGGACGGG TCGTGGACTT GAGGACCCCG CTGGCAGTCA GAAGAGAAG GGGGTTTTG
 243 K V E P K S C D K T H T C P C P A P E L L G G P S V F L F P P K P
 2401 CCAAGGACAC CCTCATGATC TCCCGAACCC CTGAGGTAC ATCGGTGGT GTGACGTGA GCCACGAAGA CCTGAGGTG AAGTCAACT GGTACGTGA
 GGTTCCTGTG GGAGTACTAG AGGCGCTGGG GACTCCAGTG TAGCACCAC CACCTGCACT CGGTGCTTCT GGGACTCCAG TTCAAGTTGA CCATGCACCT
 277 K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y V D
 2501 GCGGTGGAG GTGCATATG CCAAGACAA GCCGCGGAG GAGCAGTACA ACAGCACGTA CCGTGTGTC AGGTCCTCA CCGTCTGCA CCAGGACTGG
 GCGCACCTC CACGTATPAC GGTCTGTGTT CCGGCCCTC CTGCTCATGT TGTCTGAT GTGCAGGAGT TCGCAGGAGT GGCAGGAGT GGTCTGAGC
 310 G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W
 2601 CTGAATGGCA AGGATACAA GTGCAAGTC TCCACAAAG CCCTCCCGAG CCCCCTGAG AAAACCATCT CCAAAGCCAA AGGCGAGCCC CGAGAACCAC
 GACTTACCTT TCCTCATGTT CACGTTCCAG AGGTGTTTC GGGAGGTGG GGGGTAGCTC TTTTGGTGA GGTTCGGTT TCCGTCGGG GCTCTGTG
 343 L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q
 2701 AGGTGTACAC CTTGCCCCCA TCCCGGGAAG AGATGACCAA GAACGAGTTC AGCTGACCT GCCTGTGCTCA AGGCTTCTAT CCCAGCGACA TCGCCGTGGA
 TCCACATG GAGCGGGGT AGGCGCCCTC TCTACTGTT TCTGTGCTCAG TCGGACTGGA CGGACCACTT TCCGAAGATA GGTTCGCTGT AGCGGACCT
 377 V Y T L P P S R E E M T K N Q V S L T C L V K G F Y P S D I A V E
 2801 GTGGGAGC AATGGCAGC CGGAGAACAA CTACAAGAC ACCTCTCCG TCGTGGACTC CGAGGCTCC TTCTTCCTCT ACAGCAAGCT CACCGTGGAC
 CACCTCTCG TTACCCGTCG GCTCTGTGTT GATGTTCTGG TCGGAGGGC AGCACTCTAG GCTGCCGAGG AAGAAGAGA TGTGTTGGA GTGGCACCTG
 410 W E S N G Q P P E N M Y K T T P P V L D S D G S F F L Y S K L T V D
 2901 AAGAGCAGGT GGCAGCAGG GAACTGCTTC TCATGCTCCG TGATGCATGA GGCTCTGCAC AACCACTACA CGCAGAAGAG CCTCTCCCTG TCTCGGGTA
 TTCTGTCCA CCGTCGTCCC CTGTGCAAG AGTACGAGC ACTACGTACT CGGAGACGTG TTGTGATGT GGTCTCTCTC GGAGAGGAC AGAGGCCCAT
 443 K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G K
 3001 AATAAGCATG CGAGGCCCTT AGAGTCCCTA AGCTCCGTT GCGCGCGGC GTTTTTTATT GTTAACATCAT GTTTCACAGC TTATCATCGA TAAGCTTTAA
 TTATTCGTAC GCTGCCGGA TCTCAGGAT TCGAGCCAA CGGCGGCCCG CAAAAATAA CAATTGAGTA CAAACTGTGG AATGATGCT ATTCCGAATT
 477 O
 3101 TCGGTGAGTT TATCAGTT AAATGTCTAA CGCAGTCAGG CACGTGTAT GAAATCTAAC AATCGCTCA TGTTCATCT CCGCACCGTC ACCCTGGATG
 ACCCATCAA ATAGTGTCAA TTTAAGATT GGTCACTCC GTGGCATA CTTTAGATT TTAGCGAGT AGCAGTAGGA GCGTGGCAG TGGGACCTAC

^Start Tet Resistance Coding Sequence

FIG. 16D

3201 CTCTAGGCAT AGGCTTGGTT ATGCCGGTAC TGCCGGGCCT CTTGCGGGAT ATCGTCCATT CCGACAGCAT CGCCAGTCAC TATGGCGTGC TGCTAGCGCT
GACATCCGTA TCCGAACCAA TACGGCCATG ACGGCCCGGA GAACGCCCTA TAGCAGGTAA GGCTGTCCGTA GCGGTCAGTG ATACCGCAGC ACGATCGCGA

3301

FIG. 17A

1 GAATCAACT TCTCCATCT TTGGATBAGG AAATACAGAC ATGAAAAATC TCATGCTGA GTTGTATT TTCTTCTCT TCTCAGCTTA
 CTTAAGTTGA AGAGTAIGA AACCTATCC TTATGTCGTG TACTTTTAG AGTAACGACT CAACAATAAA TTCGAACGGG TTTTCTCTCT TCTCAGCTTA
 101 GAACGTGTG CGCAGGTAGA AGCTTTGAG ATTATCTCA CTGCAATGCT TCGCAATATG GCGCAAAATG ACCAACAGCG GTTGATTCAT CAGGTAGAGG
 CTTGACACAC CGCTCCATCT TCGAAACCTC TAATAGCAGT GACGTTACGA AGCGTTATAC CGCGTTTTAC CGCGTTTTAC CAACTAACTA GTCCATCTCC
 201 GGGCGCTGTA CGAGGTAAAG CCGCATGCCA GCATTCCTGA CGACGATPAC GAGCTGCTGC GCGATTACGT AAAGAAGTTA TTGAAGCATC CTCGTACGTA
 CCGCGCACAT GCTCCATTTT GGGCTACGGT CGTAAGGACT GCTGCTATGC CTCGACGACG CGCTAATGCA TTTCTTCAAT AACTTCGTAG GAGCAGTCAT
 301 AAAAGTTAAT CTTTTCACCA GCTGTCAATAA AGTGTACAG GCCGAGACTT ATAGTCGCTT TGTCTTTTATT TTTTAATGTA TTTGTAACTA GTACGCAAGT
 TTTTCAATTA GAAAAGTTGT CGACAGTATT TCAACAGTGC CGGCTCTGAA TATCAGCGAA ACNAAAATAA AAAATTACAT AAACATTGAT CATGCGTTCA
 401 TCACGTAAAA AGGTATCTA GAATTAAGAA GAAGATATC GCATTTCTTC TTGCACTAT TTCTGTTTT TCTATGCTA CAAACGCGTA CGCTGATATC
 AGTGCAFTTT TCCCATAGAT CTTAATACCT CTTCTTATAG CGTAAAGAAG AACGTAGATA CAAGCAAAA AGATAACGAT GTTTGCGCAT GCGACTATAG
 1 M K K N I A F L L A S M F V F S I A T N A Y A D I
 ^STII Signal TIR -1 anti-VEGF Light Chain^
 501 CAGTTGACCC AGTCCCGAG CTCCCTGTC CCCTCTGTGG GCGATAGGT CACCATCACC TGCAGCGCA GTCAAGATAT TAGCAACTAT TTAAACTGGT
 GTCAACTGGG TCAGGGCTC GAGGACAGG CCGAGACACC CGCTATCCCA GGTGTAGTG ACCTCGCTT CAGTCTATA ATCGTTGATA AATTGACCA
 26 Q L T Q S P S S L S A S V G D R V T I T C S A S Q D I S N Y L N W Y
 ^variable light (VL) cys
 601 ATCAACAGAA ACCAGGAAA GCTCCGAAAG TACTGATTTA CTTACCTCC TCTCTCACT CTGGAGTCCC TTCTCGCTC TCTGATCCG GTTCTGGAC
 TAGTTGCTT TGGTCTTTT CAGGCTTTC ATGACTAAT GAAGTGGAG AGAGAGGTGA GACCTCAGGG AAGAGCGAAG AGACCTAGG CAAGACCCCTG
 60 . Q Q K P G K A P K V L I Y F T S S L H S G V P S R F S G S G S G T
 701 GGATTTCACT CTGACCATCA GCAGTCTGA GCCAGAAGAC TTCCCAACTT ATTACTGCA ACAGTATAGC ACCGTGCCGT GGACGTTGG ACAGGTTACC
 CCTAAGTGA GACTGGTAGT CGTCAACGT CGGTCTCTG AAGGTTGAA TAATGACAGT TGTCAATCG TGGCAGGCA CCTGCAAAAC TGTCCTATGG
 93 D F T L T I S S L Q P E D F A T Y Y C Q Q Y S T V P W T F G Q G T
 ^variable light cys
 801 AAGGTGAGA TCAAAACGAAC TGTGGCTGCA CCATCTGTCT TCACTTCCC GCCATCTGAT GAGCAGTTGA AATCTGAAC TGCTTCTGTT GTGTCCTGC
 TTCCACCTCT AGTTGCTTG ACACCGACGT GGTAGACAGA AGTAGAAGG CGGTAGACTA CTGCTCACT TTAGACCTTG ACAGACAA CACACGGACG
 126 K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L L
 Constant Light cys^

FIG. 17B

901 TGAATAAAGT CTATCCACAG GAGGCCAAG TACAGTGGAA GGTGATAAC GCCCTCCAAT CGGTAACTC CCAGAGAGT GTACAGAGC AGGACAGCAA
 ACTTATTGAA GATAGGGTCT CTCGGGTTTC ATGTACACCT CCACCTATTG CGGAGAGTTA GCCCATTTAG GGTCTCTCA CAGTCTCTCG TCCTGTCTGT
 160 N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K
 1001 GGACAGCACC TACAGCCTCA GCAGCACCTT GACGCTGAGC AAAGCAGACT ACGAGAAACA CAAAGTCTAC GCCTGCEAAG TCACCCATCA GGGCCTGAGC
 CCTGTCTGG ATGTGGAGT CGTGGTGGGA CTGGGACTCG TTTCGCTGGA TGTCTTTTGT GTTTCAGATG CGGAGCTTC AGTGGTAGT CCCGGACTCG
 193 D S T Y S L S S T L T L S K A D Y E K H K V Y A C E V T H Q G L S
 ^Constant Light cys
 1101 TCGCCCGTCA CAAGAGAGCTT CAACAGGGGA GAGTGTAT TAATCCTCT AGCGGGAGC CATCTGGCG AGCTCGGTAC CCGGGATCT AGGCCTAAACG
 AGCGGGCAGT GTTCTCGAA GTTCTCCCT CTCACAATTA ATTAGGAGA TCGCGCCTGC GTAGCACCGC TCGAGCCATG GGGCCCTAGA TCCGGATTGC
 226 S P V T K S F N R G E C O
 ^cys to bind heavy
 start lambda t0 terminator^
 1201 CTCGGTTGCC GCCGGGCGTT TTTTATTGTT GCCGACGCG ATCTCGAATG AACTGTGTGC GCAGTAGAA GCTTTGGAGA TTATCGTCAC TGCAATGCTT
 GAGCCAAACG CGGCCCGGAA AAAATAACAA CGGCTGGCG TAGAGCTTAC TTGACACACG CGTCCATCTT CGAAACCTCT AATAGCAGTG ACGTTACGAA
 ^end lambda t0 terminator
 1301 CGCAATATGG CGCAAAATGA CCAACAGCGG TTGATTGATC AGGTAGAGGG GGGCTGTAC GAGTAAAGC CCGATGCCAG CATTCCTGAC GACGATACGG
 GCGTTATACC GCGTTTACT GTTGTGCGC AACTAACTAG TCCATCTCC CCGGACATG CTCCATTCG GGTACGGTC GTAAGGACTG CTGCTATGCC
 1401 AGCTGCTGG CGATTACGTA AGRAGTTAT TGAACATCC TCGTCAGTAA AAGTTAATC TTTTCAACAG CTGTCAATAA GTTGTACACG CCGAGACTTA
 TCGACGACG GCTAATGCAAT TTCTTCAATA ACTTCGTAGG AGCATCAIT TTTCAATTAG AAAAGTTGTC GACATATTT CPACAGTGCC GGCTCTGAAT
 1501 TAGTGGCTTT GTTTTATTT TTTAATGAT TGTAACTAG TACGCAAGTT CACGPAHAAA GGGTATCTAG AATTATGAAG AAGAATATCG CATTCTTCT
 ATCAGCGAAA CAAAAATAAA AAATACATA AACATTGATC ATGCGTTCAA GTGCAATTTT CCCATAGATC TTAATACTTC TTCTTATAGC GTAAAGAAGA
 M K K N I A F L L
 ^STII Signal TIR-1
 1601 TGCACTATAG TTCGTTTTT CTATTGCTAC AAACGCGTAC GCTCAGGTTT AGTGGTGA GTCTGGCGCA GAGGTGAAA AGCCAGGGG TTCAGTTAAA
 ACGTAGATAC AAGCAAAAAA GATACGATG TTTGCGCATG CGAGTCCAAG TCGACCAAGT CAGACCGCGT CTCCACTTTT TCGGTCCCG AAGTCAATTT
 10 A S M F V F S I A T N A Y A Q V Q L V Q S G A E V K K P G A S V K
 ^anti-VEGF Heavy Chain (VNERK version)
 ^Heavy Chain FR1 changed to SubgroupI consensus sequence
 1701 GTATCCTGTA AAGTTCTGG CTATACCTTC ACCAACTATG GTATAAATG GGTCCGTGAC GCGCCGGGTA AGGCGCTGGA ATGGTTGGA TGGATTAAACA
 CATAGGACAT TTCAAGACC GATATGGAAG TGGTTGATAC CATATTGAC CCAGGAGTCC CCGGGCCCAT TCCCGGACCT TACCCAACCT ACCTAATGT
 43 V S C K A S G Y T F T N Y G I N W V R Q A P G K G L E W V G W I N T
 ^Variable Heavy (VH) cys
 1801 CCTATACCGG TGAACCGACC TATGCTGCGG ATTTCAAACG TCGTTTCACT TTTTCTTTAG ACACCTCAA AAGCAGACA TACCTGCAGA TGAACAGCCT
 GGATATGGCC ACTTGGCTGG ATACGACGCC TAAAGTTTGC AGCAAGTGA AAAGAAATC TGTGAGGTTT TTCGTGCTGT ATGGAGTCT ACTTGTGGGA
 77 Y T G E P T Y A A D F K R R F T F S L D T S K S T A Y L Q M N S L

FIG. 17C

1901 GCGCGCTGAG GACACTGCGG TCTATTACTG TGCAAAAGTAC CCGCACTATT ATGTGAAGA GCGGAAGAGC CACTGTATT TCGAGCTCTG GGGTCAAGGA
 CCGCGGACTC CTGTGACGGC AGATAATGAC AGTTTCATG GCGGTGAATA TACACTTGCT CGCCTTCTCG GTGACCATAA AGCTGCAGAC CCCAGTTTCT
 110 R A E D T A V Y Y C A K Y P H Y Y V N E R K S H W Y F D V W G Q G
 ^VH cys
 2001 ACCTTGGTCA CCGTCTCCTC GGCCTCCACC AAGGCCCCAT CGGTCTTCCC CTTGGCACCC TCCTCCAAGA GCACCTCTGG GGGACACAGG GCCCTGGGCT
 TGGGACCAGT GGCAGAGGAG CCGGAGGTGG TTCCGGGTA GCCAGAAGG GAGCCGTGGG AGGAGTTCT CGTGGAGACC CCCGTCTCGC CCGGACCCCA
 143 T L V T V S S A S T K G P S V F P L A P S S K S T S G T A A L G C
 Constant Heavy 1 (CH1) cys^
 2101 GCGTGGTCA GGACTACTTC CCGGACCGG TGACGGTGC GTGGAACCTA GCGGCCCTGA CCGCGGGGT GCACACCTTC CCGGTGTTC TACAGTCTC
 CGGACCAGTT CCTGATGAAG GGGCTTGGCC ACTGCCACAG CACCTTGAT CCGCGGGACT GGTGCCGCA CGTGTGAAG GGGCCGACAGG ATGTACAGGAG
 177 L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S
 2201 AGGACTCTAC TCCTCAGCA GCGTGTGAC TGTGCCCTCT AGCAGCTTGG GCACCCAGAC CTACATCTGC AACGTGAATC ACAAGCCAG CAACACCAAG
 TCCTGAGATG AGGAGTCGT CGCACCACTG ACACGGGAGA TCGTCGAACC CGTGGGTCTG GATGTAGACG TTGCACCTAG TGTTCGGGTC GTTGTGGTTC
 210 G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K
 ^CH1 cys
 2301 GTGGACAGA AAGTTGAGCC CAAATCTGT GACAAACTC ACATATGCC ACCGTGCCA GCACCTGAAC TCCTGGGGG ACCGTGAGTC TTCTCTTTC
 CACTGTCTT TTAACCTCG GTTTAGAACA CTGTTTGAAG TGTATCGGG TGGCACGGGT CGTGGACTTG AGGACCCCC TGGCAGTCAG AAGGAGAAGG
 243 V D K K V E P K S C D K T H T C P C P A P E L L G G P S V F L F P
 ^cys to bind light chain ^hinge cys
 2401 CCCCAAAACC CAAGGACACC CTCATGATCT CCGGACCCC TGAGTCACTA TGGTGTGG TGGACGTGAG CCACGAGAGC CCTGAGGTCA AGTTCAACTG
 GGGGTTTGG GTTCTGTGEG GAGTACTAGA GGGCCCTGGG ACTCCAGTGT ACGCACACC ACCGTGCACTC GTGTCTTCTG GGAATCCAGT TCAAGTTGAC
 277 P K P K D T L M I S R T P E V T C V V D V S H E D P E V K F N W
 ^Constant Heavy 2 (CH2) cys
 2501 GTAGTGGAC GCGGTGAGG TGCATAATGC CAAGACAAAG CCGCGGGAGG AGCAGTACAA CAGCACGTAC CGTGTGTTCA CCGTCTTCAC CGTCTTCAC
 CATGCACCTG CCGCACCTCC AGTATPACG GTTCTGTTTC GTTCTGTTTC GCGGCCCTCC TCGTCACTGT GTGCTGCATG GCACACCAAGT CGCAGGAGTG GCAGGACGTG
 310 Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H
 2601 CAGGACTGCG TGAATGGCAA GGAGTACAAG TGCAAGTCT CCAACAAAG CCTCCAGCC CCGCATCGAGA AAACCATCTC CAAAGCCAAA GGGCAGCCCC
 GTCTGTACCG ACTTACCGTT CCTCATGTC ACGTTCAGA GTTGTGTTCC GAGGAGTCGG GGTGAGTCTT TTGTGTAGAG GTTTCGGTTT CCCGTGGGG
 343 Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R
 ^CH2 cys
 2701 GAGAACCA GGTGTACACC CTGCCCCCAT CCGGGAAGA GATGACCAAG AACCAGGTCA GCGTGACCTG CCTGGTCAAA GGTCTTCTATC CCAGGACAT
 CTCTTGGTGT CCACATGAG GACGGGGTA GGGCCCTTCT CTACTGGTTC TTGTCCAGT CCGACTGGAC GGAACAGTTT CCGAAGATAG GGTTCGTGTA
 377 E P Q V Y T L P P S R E E M T K N Q V S L T C L V K G F Y P S D I
 ^Constant Heavy 3 (CH3) cys

FIG. 17D

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2801 CGCGGTGGAG TGGGAGAGCA ATGGGAGGCC GGAGACAAAC TACAAGACCA CGCCTCCCGT GCTGGACTCC GACGGCTCCT TCTTCTCTA CAGCAAGCTC
    GCGGACCTC ACCCTCTCGT TACCCCTCGG CCTCTGTGTG ATGTTCTGGT GCGAGGGCA CGACCTGAGG CTGCCGAGGA AGAAGGAGAT GTCGTTCCGAG
410 A V E W E S N G Q P E N N Y K T T P P V L D S D G S F L Y S K L
2901 ACCGTGGACA AGAGCAGGTG GCAGCAGGGG AACGTCTTCT CATGCTCCGT GATGCATGAG GCTCTGCACA ACCACTACAC GCAGAAGAGC CTCTCCCTGT
    TGGCACCTGT TCTCGTCCAC CFTCGTCCAC GTACGAGGCA CTAGCTACTC CGAGACGCTG TGGTGAATG CTTCTTCTCG GAGAGGGACA
443 T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S
    ^CH3 cys
3001 CTCGGGTAA ATAAGCATCC GACGGCCCTA GAGTCCCTAA CGCTCGGTTG CGCGGGGCG TTTTITATTG TTGACAGCT TTTGACAGCT TATCATCGAT
    GAGGCCATT TATTGCTACG CTGCCGGCAT CTCAGGATT GCGAGCCAAC GGCGGCCCGC AAAAATAAC AATTGAGTAC AACTGTGCA ATAGTAGCTA
477 P G K O ^start lambda t0 terminator ^end lambda t0 terminator ^start of tet resistance promoter
    ^-35 of promoter
3101 AAGCTTTAAI GCGGTAGTTT ATCAGAGTTA AATTGCTAAC GCAGTCAGGC ACCGTGTATG AAATCTAACA ATGGCTCAT CGTCATCCTC GGCACCGTCA
    TTCGAATTA CGCCATCAAA TACGTCAAT TTAACGATTG CGTCAGTCCG TGGCACATAC TTITAGATGT TACGCGAGTA GCAGTAGGAG CCGTGGCAGT
    ^-10 region of tet resistance promoter ^start of tet resistance translation
3201 CCCTGGATGC TGTAGGCATA GGTCTGGTTA TCGCGGTACT GCGGGGCTC TTGGGGGATA TCGTCCATTC CGACAGCATC GCCAGTCACT ATGGCGTGCT
    GGGACCTACG ACATCGTAT CCGAACCAAT ACGGCCATGA CGGCCCGGAG AACGCCCTAT AGCAGGTAAG GCTGTCTGAG CCGTCAGTGA TACCGCACGA
3301

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FIG. 18A

1 GAATTCACCT TCTCCATCT TTGGATAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA GTTGTATTTT AGCTTGGCC AAAAGAAGA AGAGTCGAAT
 CTTAAGTTGA AGAGGTATGA AACCTATTC TTTATGTCTG TACTTTTAG AGTAACGACT CAACAATAAA TTCTTCTTCT TCTCAGCTTA
 101 GAACTGTGCG CGCAGGTAGA AGCTTTGAG ATTATCGTCA CTGCAATGCT TCGCAATATG GCGCAAAATG ACCAACACGG GTTGATTGAT CAGGTAGAGG
 CTTGACACAC GCGTCCATCT TCGAAACCTC TAATAGCAGT GACGTACGA AGCTTATAC CCGGTTTTTAC TGGTTGTGCG CAACATAACTA GTCCATCTCC
 201 GGGCGCTGTA CGAGGTAAAG CCCATGCTCA GCATTCTGA CGACATACG GAGCTGTGCG GCGATTACGT AAAGAAGTTA TTGAAGCATC CTCGTGAGTA
 CCGCGACAT GCTCCATTTC GGGCTACGGT CGTAGGACT GCTGCTATGC CTCGACGACG CGCTAATGCA TTTCTTCAAT AACTTCGTAG GAGCAGTCAT
 301 AAAAGTTAAT CTTTCAACA GCTGTCAATA AGTTGTACG CGGCTCTGAA TATCAGCGAA ACAAAAATAA AAAATTACAT AAACATTGAT CATGCGTTCA
 TTTTCAATTA GAAAAGTTGT CGACGTATT TCAACAGTGC GCGCTCTGAA TATCAGCGAA ACAAAAATAA AAAATTACAT AAACATTGAT CATGCGTTCA
 401 TCACGTAAAG AGGGTATCTA GAAATTATGA GAAGAATATC GCATTCTTTC TTGCATCTAT GTTCGTTTTT TCTATTGCTA CAAACGCGTA CGTGAATATC
 AGTGCATTTT TCCCATAGAT CTTAATACCTT CTCTCTATAG CGTAAAGAAG AACGTAGATA CAAGCAAAA AGATAACGAT GTTTGGCAT GCGACTATAG
 1 M K K N I A F L L A S M F V F S I A T N A Y A D I
 ^STII Signal TIR -1
 anti-VEGF Light Chain^
 501 CAGTTGACCC AGTCCCGGAG CTCCTCTGTC GCGATAGGT CACCATCACC TGCAGCGCAA GTCCAGGATAT TAGCAACTAT TTAACCTGGT
 GTCAACTGGG TCAGGGGCTC GAGGGACAGG CGGAGACACC CGCTATCCCA GTGTAGTGG ACCTCGCGTT CAGTCCCTATA ATCGTTGATA AATTGACCA
 26 Q L T Q S P S S L S A S V G D R V T I T C S A S Q D I S N Y L N W Y
 ^variable light (VL) cys
 601 ATCAACAGAA ACCAGGAAA GCTCCGAAAG TACTGATTA CTTCACCTCC TCTCTCCACT CTGGAGTCCC TTCTGGCTTC TCTGGATCCG GTTCTGGGAC
 TAGTTGTCTT TGTCCCTTTT CGAGCTTTC ATGACTAAAT GAAGTGGAGG AGAGAGGTGA GACCTCAGG AAGAGCGAAG AGACCTAGGC CAAGACCCCTG
 60 Q Q K P G K A P K V L I Y F T S S L H S G V P S R F S G S G S G T
 701 GGATTTCACT CTCACCATCA GCAGTCTGCA GCCAGAGAC TTGCGCAACT ATTACTGTCA ACAGTATAGC ACCGTGCGGT GGACGTTTGG ACAGGTATCC
 CCTAAGTGA GACTGGTAGT CFTCAGAGGT CGGTCTCTG AAGCGTTGAA TAATGACAGT TGTATATCG TGGCAGCGCA CCGTCAAAAC TGTCCCATGG
 93 D F T L T I S S L Q P E D F A T Y Y C Q Q Y S T V P W T F G Q G T
 ^Variable light cys
 801 AAGGTGGAGA TCAAAACGAAC TGTGGCTGCA CCATCTGTCT TCATCTTCCC GCCATCTGAT GAGCAGTTGA AATCTGGAAC TGCTTCTGTT GTGTGCTGC
 TTCCACCTCT AGTTTGCTTG ACACCGAGCT GGTAGACAGA AGTAGAAGGG CCGTAGACTA CTCGTCAACT TTAGACCTTG ACGAAGACAA CACACGAGC
 126 K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L L
 Constant light cys^

FIG. 18B

901 TGAATAACTT CTATCCCGA GAGGCCAAG TACAGTGAA GGTGGATAAC GCCCTCCAAT CGGTAACATC CCAGAGAGAT GTACACAGAG AGACAGCAA
 ACTTATATGAA GATAGGGTCT CTCGGGTTC ATGTCACTT CCACCTATTG CGGAGGTTA GCCATTGAG GGTCTCTCA CAGTGTCTCG TCCTGTGTT
 160 N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K
 1001 GGACAGCACC TACAGCCTCA GCAGCAACCT GACGCTGAGC AAAGCAGACT ACGAGAAACA CAAAGTCTAC GCTGCGAG TCACCCATCA GGGCCTGAGC
 CCTGCTGAG ATGTCGGAGT CGTCGTGGA CTGCGACTCG TTTCGTCTGA TGCTCTTTGT GTTTCAGATG CGGACGCTTC AGTGGTAGT CCCGGAATCG
 193 D S T Y S L S S₁ T L T L S K A D Y E K H K V Y A C E V T H Q G L S
 ^Constant Light cys
 1101 TCGCCCGTCA CAAAGAGCTT CAACAGGGA GAGTGTAAAT TAAATCCTCT AGCCCGGAG CATCGTGGG AGCTCGGTAC CCGGGGATCT AGGCCTAACG
 AGCGGGCAGT GTTCTCGAA GTTCTCCCT CTCACAAATTA ATTAGGAGA TCGGCGCTGC GTAGCACCGC TCAGAGCCATG GGGCCCTAGA TCCGGATTGC
 226 S P V T K S F N R G E C O
 ^cys to bind heavy
 start lambda t0 terminator^
 1201 CTCGGTTGCC GCGGGCGT TTTTATGTT GCGGACGCG ATCTCGAATG AACTGTGTC GCAGGTAGAA GCTTGGAGA TTATCGTCAC TGCAATGCTT
 GAGCCAAACG CGGCCCGCAA AAAATAACAA CCGCTGCGG TAGAGCTTAC TTGACACACG CGTCCATCTT CGAAACCTCT AATAGCAGTG AGTTACGAA
 ^end lambda t0 terminator
 1301 CGCAATATGG CGCAAAATGA CCAACAGCGG TTGATTGATC AGGTAGAGG GCGCTGTATC GAGGTAAAGC CCGATGCCAG CATTCCTGAC GACGATACGG
 GCGTTATACC GCGTTTACT GGTGTGCGC AACTAACTAG TCCATCTCC CCGCGACATG CTCCTATTTC GGTACGCTC GTAAGGACTG CTGCTATGCC
 1401 AGCTGCTGG CGATTACGTA AAGAAATTAT TGAAGCATCC TCGTCAGTAA AAAGTTAATC TTTTCAACAG CTCTCATAAA GTTGTACCG CCGAGACTTA
 TCGACGACGC GCTAATGCAAT TTCTTCAATA ACTTCGTAGG AGCAGTCAAT TTTCATATAG AAAAGTTGTC GACAGTATTT CAACAGTGCC GGTCTCTGAAT
 1501 TAGTCGCTTT GTTTTATTT TTTAATGTAAT TGTAACTAG TACGCAAGTT CACGTAAAAA GGGTATCTAG AATTATGAAG AAGAATATCG CATTTCTTCT
 ATCAGCGAAA CAAAAATAAA AAATTACATA AACATTGATC ATGCGTTCAA GTGCAATTTT CCCATAGATC TTAATACCTC TTCTTATAGC GTAAAGAAGA
 M K K N I A F L L
 ^STII Signal TIR-1
 1601 TGCATCTATG TTCTTTTTTT CTATGCTTAC AAACGGGTAC GCTCAGTTC AGCTGCAAGA GTCTGGCCCG GGCTGGTGA AACCATCTCA GACTCTCTCC
 ACGTAGATAC AAGCAAAAAA GATAACGATG TTTCGCGATG CGAGTCCAAG TCGAGCTTCT CAGACCCGGC CCGGACCACT TTGGTAGAGT CTGAGAGAGG
 10 A S M F V F S I A T N A Y A Q V Q L Q E S G P G L V K P S Q T L S
 ^anti-VBGF heavy chain (VNERK version)
 ^Heavy chain FR1 changed to Subgroup II consensus sequence
 1701 TTGACTTGTA CTGTTCTCG CTATACCTTC ACCAACTATG GTATAAACTG GGTCCGTAC GCGCCGGTA AGGCGCTGGA ATGGGTGGA TGGATTAAACA
 AACTGAACAT GACAAAGACC GATATGGAAG TGGTTGATAC CATATTGAC CCAGGAGTC CCGGGCCCAT TCCCGGACCT TACCCAACT ACCTAATTGT
 43 L T C T V S G Y T F T N Y G I N W V R Q A P G K G L E W V G W I N T
 ^Variable Heavy (VH) cys
 1801 CCTATACCG TGAACCGACC TATGCTGGG ATTTCAAAG TGGTTTCACT TTTTCTTAG ACACCTCCAA AAGCAGACGA TACCTGCAGA TGAACAGCCT
 GGATATGCC ACTTGGCTGG ATACGACGCC TAAAGTTGC AGCAAGTGA AAAAGAAATC TGTGAGGTT TTCTGTCTGT ATGACGCTCT ACTTGTGGA
 77 Y T G E P T Y A A D F K R R F T F S L D T S K S T A Y L Q M N S L

FIG. 18C

1901 GCGGGCTGAG GAACTGCGG TCTATTACTG TGCAGAGTAC CCGCACTATT ATGTGAACGA GCGGAAGAGC CACTGGTATT TCGACGTCTG GGTCAAGGA
 CCGCGCACTC CTGTGACGGC AGATAATGAC ACCTTTTCATG GCGGTGATPA TACACTTGCT CGCTTCTCG GTGACCATAA AGTGCAGAC CCAGTTCTT
 110 R A E D T A V Y Y C A K Y P H Y Y V N E R K S H W Y F D V W G Q G
 ^VH cys
 2001 ACCCTGGTCA CCGTCTCTC GGCCTCCACC AAGGGCCCAT CCGTCTTCCC CTTGGCACCC TCCTCAAGA GCACCTCTGG GGCACACAGG GCCTGGGCT
 TGGACCACTT CCGTCTCTC GGCCTCCACC AAGGGCCCAT CCGTCTTCCC CTTGGCACCC TCCTCAAGA GCACCTCTGG GGCACACAGG GCCTGGGCT
 143 T L V T V S S A S T K G P S V F P L A P S S K S T S G G T A A L G C
 Constant Heavy 1 (CH1) cys
 2101 GCTTGTGTC GAACTACTTC CCGCAACCGG TGAGGGTCTC GTGGAATCA GCGGCCCTGA CCAGGGCGGT GCACACCTTC CCGCTGTCTC TACAGTCTCT
 CCGACCACTT CCGTCTCTC GGCCTCCACC AAGGGCCCAT CCGTCTTCCC CTTGGCACCC TCCTCAAGA GCACCTCTGG GGCACACAGG GCCTGGGCT
 177 L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S
 2201 AGGACTCTAC TCCCTCAGCA CCGTGTGTGAC TGTGCCCTCT AGCAGCTTGG GCACCCAGAC CTACATCTGC AACGTGATC ACAAGCCCAG CAACACCAAG
 TCCCTGAGATG AGGAGTCTGT CCGACCACTG ACACGGGAGA TCGTCGAACC CCGTGGTCTG GATGTAGACG TTGCACCTAG TGTTCGGGTC GTTGTGGTTC
 210 G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K
 ^CH1 cys
 2301 GTGGACAGA AAGTTCAGCC CAAATCTTGT GACAAATCTC ACACATGCC ACCGTGCCA GCACCTGAAC TCCTGGGGGG ACCCTCAGT TCCTCTTCTC
 CACCTGTCTT TCAACTCGG GTTTAGACA CTGTCTTGG TGTGTACGG TGGCACGGGT CGTGGACTTG AGGACCCCC TGGCAGTCTG AAGGAGAGG
 243 V D K K V E P K S C D K T H T C P P C P A P E L L G G P S V F L F P
 ^cys to bind light chain ^hinge cys
 2401 CCCCCAACC CAAGGACACC CTCATGATCT CCGGACCCC TGAGGTCA CAGCACTGAG CCACGAAGAC CCTGAGGTCA AGTTCACATG
 GGGGTTTGG GTTCTGTGTG GAGTACTGAG GGGCTGGGG ACTCCAGTGT AGCACCACC ACCTGCACTC GTTGTCTCTG GGAATCCAGT TCAAGTTGAC
 277 P K P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W
 ^Constant Heavy 2 (CH2) cys
 2501 GTACGTGGAC GCGGTGGAGG TGCAATATGC CAAGACAAG CCGCGGGAGG AGCAGTACA CAGCACTGAC CCGTGTGCTA CCGTCTCTAC CGTCTGTCAC
 CATGCACTG CCGCACTTC ACCTATACG GTTCTGTCTT CCGGCCCCCTC TGTCTATGTT GTCTGTGATG GCACACCACT CCGCAGGAGT GCAGGACCTG
 310 Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H
 2601 CAGGACTGGC TGAATGGCAA GAGTACAA GTCAGGTCT CCAACAAGC CTTCCAGCC CCCATCGAGA AAACCATCTC CAAAGCCAAA GGCAGAGCCC
 GTCTGACCG ACTTACCGT CTTCAATGTC ACCTTCCAGA GTTGTGTTG GAGGGTCTG GGGTAGCTCT TTGTGTAGAG GTTTCGGTTT CCGTGGGGG
 343 Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R
 ^CH2 cys
 2701 GAGAACCACA GGTGTACACC CTGCCCCCAT CCGGGGAAGA GATGACCAAG AACCAGTCA GCCTGACCTG CCTGTCTCAA GGCCTTCTATC CCAGCGACAT
 CTCTTGTGT CCACATGTGG GACGGGGTA GGGCCCCCTT CTACTGTGTC TTGTCCAGT CCGACTGAGC GACCACTT CCGAGATAG GGTGCTGTGA
 377 E P Q V Y T L P P S R E E M T K N Q V S L T C L V K G F Y P S D I
 ^Constant Heavy 3 (CH3) cys

FIG. 18D

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2801 CGCGGTGGAG TGGGAGAGCA ATGGCAGCC GGAGAACAC TACAAGACCA CGCCTCCCGT GCTGGACTCC GACGGTCCCT TCTTCTCTTA CAGCAAGCTC
GGGCACCTTC ACCCTCTCGT TACCCGTCGG CCTCTGTGTG ATGTTCTGTT GCGGAGGGCA CGACCTGAGG CTGCCGAGGA AGAAGAGAT GTCGTTGAG
410 A V E W E S N G Q P E N N Y K T T P P V L D S D G S F L Y S K L
2901 ACCGTGGACA AGAGCAGGTG GCAGCAGGGG AACCTCTTCT CATGCTCCCT GATGCATGAG GCTCTGCACA ACCACTACAC GCAGAAGAGC CTCTCCCTGT
TGGCACCTGT TCTCGTCCAC CGTCGTCCCC TTGCAGAAGA GTACGAGGCA CTACGTACTC CGAGACCTGT TGGTGATGTG CGTCTTCTCG GAGAGGGACA
443 T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S
^CH3 cys
3001 CTCGGGGTAA ATAAGCATGC GACGGCCCTA GAGTCCCTAA CGTTCGGTTG CCGCCGGGGG TTTTATTG TTAACATG TTTGACAGCT TATCATCGAT
GAGGCCCAT TATTCTGTACG CTGCCGGGAT CTCAGGGATT GCGAGCCAAC GCGGCCCGC AAAAATAAAC AATTGAGTAC AAACCTGTCTGA ATAGTAGCTA
477 P G K O ^start lambda t0 terminator ^end lambda t0 terminator ^start of tet resistance promoter
^start of promoter
3101 AAGCTTTAT GCGGTAGTTT ATCAGATT AATTGCTAAC GCAGTCAGGC ACCGTGTATG AAATCTAACA ATGCGTTCAT GGTATCCTC GGCACCGTCA
TTCCAAATTA CGCCATCAAA TAGTGTCAAT TTAACGATTG CGTCAGTCCG TGGCACAATC TTTAGATTGT TAGCCGAGTA GCAGTAGGAG CCGTGGCAGT
^start of tet resistance translation
^start of tet resistance promoter
3201 CCCTGGATEC TGTAGGCATA GGTCTGTGTTA TGCCGTTACT GCCGGGCTC TTGCGGGATA TCGTCCATTC CGACAGCATC GCCAGTCACT ATGGCGTGCT
GGGACCTACG ACATCCGTAT CGAACCAAT ACGGCCATGA CCGCCGGAG AACGCCCTAT AGCAGGTAAG GCTGTCTGTAG CGGTCTAGTGA TACCGCACGA
3301

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FIG. 19A

1 GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAATAATC TCATTGCTGA GTTCTTATTT AAGCTTGCC AAAAAGAAGA AGAGTCGAAT
 CTTAAGTTGA AGAGGTATGA AACCTATTC TTTATGCTG TACTTTTAG AGTAACGACT CAACAATAAA TTGGAACGGG TTTTCTTCT TCTCAGCTTA
 101 GAACGTGTG CGCAGGTAGA AGCTTTGGAG ATTATCTCA CTGCAATGCT TCGCAATATG GCGCAAAATG ACCAACACGG GTTGATTGAT CAGGTAGAGG
 CTTGACACAC GCGTCCATCT TCGAAACCTC TAATAGCAGT GACGTTACGA AGCGTTATAC CGCGTTTTAC TGGTGTGCG CAACTAATA GTCCATCTCC
 201 GGGCGCTGTA CGAGGTAAAG CCGATGCCA GCATTCCTGA CGACGATACG GAGTGTCTGC GCGATTACGT AAAGAAGTTA TTGAAGCATC CTCGTCASTA
 CCCGCGACAT GCTCCATTTT GGGCTACGGT CETHAGGACT GCTGCTATGC CTCGACGACG CGCTAATGCA TTTCTTCAAT AACTTCGTAG GAGCAGTCAT
 301 AAAAGTTAAT CTTTTCACA GCTGTCATAA AGTTGTACG GCCGAGACTT ATAGTCGCTT TGTTTTATTT TTTTAATGTA TTGTAACTA GTACGCAAGT
 TTTTCATTA GAAAGTTGT CGACAGTATT TCACAACGTGC CGGCTCTGAA TATCAGCGAA ACAAAATAA AAAATTACAT AAACATTGAT CATGCGTTCA
 401 TCACGTAAA AGGTATCTA GAATTAATGAA GAAGAATATC GCATTTCTTC TTGCATCTAT GTTCGTTTTT TCTATGCTA CAAACGCGTA CGCTGATATC
 AGTGCATTTT TCCCATAGAT CTTAATACTT CTTCTTATAG CGTAAGAAG AACGTAGATA CAAGCAAAA AGATAACGAT GTTTGCGCAT GCGACTATAG
 1 M K K N I A F L L A S M F V F S I A T N A Y A D I
 Anti-VEGF Light chain (version Y0317)[^]
 1 ^STII Signal TIR -1
 501 CAGTTGACCC AGTCCCCGAG CTCCTGTGTC GCATAGGGT CACCATCACC TGCAGCGCAA GTCAAGATAT TAGCAACTAT TTAAACTGGT
 GTCAACTGGG TCAGGGGCTC GAGGACACAG CGGAGACACC CGCTATCCCA GTGGTAGTGG ACGTGCGGTT CAGTCTATA ATCGTTGATA AATTGACCA
 26 Q L T Q S P S S L S A S V G D R V T I T C S A S Q D I S N Y L N W Y
 601 ATCAACAGAA ACCAGGAAA GCTCCGAAAG TACTGATTTA CTTACCTCC TCTCTCACT CTGGAGTCCC TTCTCGCTTC TCTGATCCG GTTCTGGGAC
 TAGTTGCTTT TGGTCCCTTT CGAGGCTTTC ATGACTAAAT GAAGTGGAGG AGAGAGGTGA GACCTCAGGG AAGAGCGGAG AGACCTAGGC CAAGACCCGTG
 60 Q Q K P G K A P K V L I Y F T S S L H S G V P S R F S G S G T
 701 GGATTTCACT CTGACCATCA GCAGTCTGCA GCGAGAGAC TTGCAACTT ATTACTCA ACAGTATAGC ACCGTGCGGT GGAGTTTGG ACAGGGTACC
 CTTAAAGTGA GACTGGTAGT CGTCAGACGT CGGTCTTCTG AAGCGTTGAA TAATGACAGT TGTCAATCG TGGCAGGGA CCTGCAAAACC TGTCCTCATGG
 93 D F T L T I S S L Q P E D F A T Y Y C Q Q Y S T V P W T F G Q G T
 801 AAGGTGAGA TCAACGAAC TGTGGCTGCA CCACTGTCTT TCATCTTCCC GCATCTGAT GAGCAGTTGA AATCTGGAAC TGTCTCTGTT GTGTGCTGTC
 TTCCACCTCT AGTTTGCTTG ACACCGACGT GGTAGACAGA AGTAGAAGGG CCGTAGACTA CTCGTCAACT TTAGACCTTG ACGAAGACAA CACACGGAGC
 126 K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L L
 901 TGAATAACTT CTATCCAGA GAGGCCAAG TACAGTGGAA GGTGGATAAC GGCCTCCAAT CCGGTAACTC CCAGGAGAGT GTCACAGAGC AGGACAGCAA
 ACTTATGAA GATAGGTTCT CTCCGGTTTC ATGTACCTT CCACCTATTG CCGGAGGTTA GCCCATGAG GGTCTCTCTCA CAGTGTCTCG TCCTGTCTGTT
 160 N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K

FIG. 19B

1001 GGACAGCACC TACAGCCTCA GCAGCACCCCT GAGCGTGAGC AAAGCAGACT ACAGAAACA CAAAGTCTAC GCCTGCGAAG TCACCCATCA GGGCCTGAGC
 CCTGTCTGCG AGTCTCGAGT CGTCTGCGGA CTGCGACTCG TTTCGTCTGA TGCTCTTTGT GTTTCAGATG CCGACGCTTC AGTGGTAGT CCCGACTCG
 193 D S T Y S L S S T L T L S K A D Y E K H K V Y A C E V T H Q G L S
 1101 TCGCCCGTCA CAAAGAGCTT CAACAGGGGA GAGTGTAAAT TAAATCCTCT AGCCCGAGC CATCTGCGG AGTCGGTAC CCGGGATCT AGGCTAAACG
 AGCGGGCAGT GTTCTCGAA GTTGTCCCTT CTCAAAITTA ATTAGGAGA TCGCGCCTGC GTAGCACCGC TCGAGCCATG GGGCCCTAGA TCCGGATTGC
 226 S P V T K S F N \ R G E C O
 1201 CTCGGTTGCC GCGGGCGGTT TTTTATTTT GCGAGCGGC ATCTCGAATG AACTGTGTGC GCAGGTAGAA GCTTTGGAGA TTATCGTCAC TGCAATGCTT
 GAGCCACACG CCGCCCGCAA AAAATAACAA CCGCTGCGG TAGAGCTTAC TTACACACG CGTCCATCTT CGAACCTCT AATAGCAGTG ACGTTACGAA
 1301 CGCAATATGG CGCAAAATGA CCAACNGCGG TTGATTTGATC AGGTAGAGGG GCGGCTGTAC GAGGTAAAGC CCGATGCCAG CATTCCTGAC GACGATACGG
 GCGTTATACC GCGTTTACT GGTGTGCGC AACTAATAG TCCATCTCCC CCGCGACATG CTCCATTTCG GGTACGGTC GTAAGGACTG CTGCTATGCC
 1401 AGCTGCTGCG CGATTACGTA AAGAGTTAT TGAAGCATCC TCGTCAGTAA AAAGTTAATC TTTTCAACAG CTGTCAATAA GTTGTACACG CCGAGACTTA
 TCGACGACGC GCTAATGCAT TTCTTCAATA ACTTCGTAGG AGCAGTCAIT TTTCATTAG AAAAGTTGTC GACAGTATTT CAACAGTGCC GGTCTGTAAT
 1501 TAGTGCCTTT GTTTTATTT TTTAATGTAT TTGTAATGAT TACGCAAGTT CACGTAAAAA GGGTATCTAG AATTATGAAG AAGAATATCG CATTTCTTCT
 ATCAGCGAAA CAAATAATAA AAATTACATA AACATTGATC ATCGGTTCAA GTGCATTTT CCCATAGATC TTAATACTTC TTCTTATAGC GTAAAGAAGA
 1 M K K N I A F L L
 ^STII Signal TIR-1
 1601 TGCATCTATG TTCTGTTTTT CTAFTGCTAC AAACGCGTAC GCTCAGGTTG AGTGTGCA GTCTGGCGCA GAGTAAAA AGCCAGGGC TTCAGTTAAA
 ACGTAGATAC AAGCAAAAAA GATAACGATG TTTCGCGATG TCGATCCAAG TCGACACAGT CAGACGGCGT CTCACITTTT TCGGTCCCGG AAGTCAATTT
 10 A S M F V F S I A T N A Y A Q V Q L V Q S G A E V K K P G A S V K
 ^Anti-VEGF (version Y0317) Heavy Chain
 ^Heavy Chain FR1 changed to SubgroupI consensus sequence
 1701 GTATCTTGTA AAGCTTCTGG CTACGACTTC ACGCATTACG GTATGAACTG GGTCCGTACG GCCCGGGTA AGGCCTGGA ATGGTTGGA TGGATTAA
 CATAGACAT TTCGAAGACC GATGCTGAAG TCGGTGATGC CATACTTGAC CCAGGAGTC CCGGGCCCAT TCCCGACCT TACCAACCT ACCTAATGT
 43 V S C K A S G Y D F T H Y G M N W V R Q A P G K G L E W V G W I N T
 1801 CCTATACCGG TGAACCGACC TATGCTGCGG ATTTCAAACG TCGTTTCACT TTTTCTTTAG ACACCTCCAA AAGCACAGCA TACCTGCGAGA TGAACAGCCT
 GGAATGCGC ACTTGGCTGG ATACGACGCC TAAAGTTGCG AGCAAGTGA AAAAATAATC TGTGAGGTT TTCTGTCTCT ATGACGCTCT ACTTGTGCGA
 77 Y T G E P T Y A A D F K R R F T F S L D T S K S T A Y L Q M N S L
 1901 GCGCGCTEAG GACACTGCCG TCTATTACTG TCAAAATAC CCGTACTATT ATGGAGCAGG CCACTGGTAT TTGACGCTT GGGTCAAGG AACCTGGTC
 CGCGGACTC CTGTGACGCG AGATAATCAC AGTTTTCATG GGCATGATAA TACCTGCTC GTGACCATTA AAGCTGCAGA CCCAGTTCC TTGGGACCAG
 110 R A E D T A V Y Y C A K Y P Y Y Y G T S H W Y F D V W G Q G T L V
 2001 ACCGCTCTCT CGGCTCTCAC CAAAGGCGCA TCGGTCTTCC CCCTGACCC CTCCTCCAAG AGCACTCTG GGGCACACAG GGGCCTGGGC TGCCTGGTCA
 TGGCAGAGA GCGGAGGTE GTTCCCGGCT AGCCAGAGG GGGACGCTGG GAGGAGTTTC TCGTGGAGAC CCGGTGTGCG CCGGACCCG ACGGACAGT
 143 T V S S A S T K G P S V F P L A P S S K S T S G G T A A L G C L V K

FIG. 19C

2101 AGGACTACTT CCGCGAACCG GTGACGGTGT CGTGGAACTC AGGCGCCCTG ACCAGCGGGG TGCACACACCTT CCGGGCTGTC CTACAGTCCT CAGGACTCTA
 177 TCCTGATGAA GGGGCTTGGC CACTGCCACA GCACCTGAG TCCGCGGGAC TGGTGGCCGC ACCTGTGAAA GGGCCGACAG GATGTGAGG GTCCTGAGAT
 D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S G L Y
 2201 CTCCTCAGC AGCGTGGTGA CTGTGCCCTC TAGCAGCTTG GGCACCCAGA CCTACATCTG CAACGTGAT CACAAGCCCA GCAACACCAA GGTGGACAAG
 GAGGAGTGG TCGCACCACT GACACGGGAG ATCGTGAAC CCGTGGGTCT GGATGTAGAC GTTGACATTA GTGTGCGGT CGTTGTGGTT CCACCTGTTC
 210 S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K
 2301 AAGCTGAGC CCAATCTTG TCACAAACT CACATGCC CACCGTCCC AGCACTGAA CTCTGGGG GACCGTCAGT CTTCCTCTTC CCCCACAAAC
 TTTCACCTG GGTTAGAAC ACTGTTTGA GTGTGACGG GTGGACCGGG TCGTGACTT GAGACCCCC CTGGCAGTCA GAAGGAGAAG GGGGTTTGG
 243 K V E P K S C D K T H T C P P C P A P E L L G G P S V F L F P P K P
 2401 CCAGGAGAC CCTCATGATC TCCCGACCC CTGAGTCACT ATGGTGGTG GTGGACGTGA GCCACGAGA CCTGAGGTG AAGTCAACT GGTACGTGGA
 GGTTCCTCTG GGAGTACTAG AGGGCTGGG GACTCCAGTG TACGACCCAC CACTGCACT CCGTGCCTCT GGGACTCCAG TTCAAGTTGA CCATGCACCT
 277 K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y V D
 2501 CGGCGTGAG GTGCATAATG CCAAGACAA GCCGCGGGAG GAGCAGTACA ACAGCACGTA CCGTGTGTC AGCTCTCTCA CCGTCTCTGA CCAGGACTGG
 GCGCACCTC CAGGTATTAC GGTCTCTTT CGGCGCCCTC CTCGTCATGT TGTGTCATGT GGCACACAG TCGCAGAGT GGCAGGACGT GGTCTTGACC
 310 G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W
 2601 CTGAATGCA AGGAGTACAA GTGCAAGTTC TCCACAAAG CCTTCCCAGC CCCATCGAG AAAACCATCT CCAAGGCCAA AGGGCAGCCC CGAGAACCAC
 GACTTACGT TCCTCATGTT CACGTTCCAG AGGTGTTTC GGGAGGGTGG GGGGTAGTCT TTTTGGTGA GGTTCGGTT TCCCGTCGGG GCTCTTGGTG
 343 L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q
 2701 AGGTGTACAC CCGTCCCCCA TCCCGGGAAG AGATGACCAA GAACCAAGTC AGCTGACCT GCCTGTGCAA AGGCTTCTAT CCCAGGACA TCGCGGTGGA
 TCCACATGT GGCAGGGGT AGGGCCCTTC TCTACTGGTT CTGGTCCAG TCGGACTGGA CGGACCACTT TCCGAAGATA GGTGCGCTGT AGCGCACCT
 377 V Y T L P P S R E E M T K N Q V S L T C L V K G F Y P S D I A V E
 2801 GTGGGAGAGC AATGGGAGC CGGAGAACAA CTACAAGACC ACGCTCCC GGTGTGACTC CGACGGTCC TTCTTCTCT ACAGCAAGCT CACGTGGAC
 CACCTCTCG TTACCGCTCG GCCTCTTGT GATGTTCTGG TGGCGAGGC ACACCTGAG GCTGCCGAGG AAGAGGAGA TGTCTGCGA GTGGCACCTG
 410 W E S N G Q P E N N Y K T T P P V L D S D G S F F L Y S K L T V D
 2901 AAGAGCAGGT GGCAGCAGG GAACGTCTTC TCATGCTCG TGATGCAIGA GGCTCTGCAC AACCCTACA CGCAGAAGAG CCTCTCCCTG TCCTCGGGTA
 TTCTCGTCCA CCGTCTCCC CTTCGAGAAG AGTACGAGGC ACTACGTA CTGAGAGCTG CGGAGAGCTG TTGGTGATGT CGGTCTCTC GGAGAGGAC AGAGGCCCAT
 443 K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G K
 3001 AATAAGCATG CGACGGCCCT AGAGTCCCCTA ACGTCTGGT GCGCCCGGGC GTTTTATTT GTTAATCAT GTTTGACAGC TTATCATCGA TAAGCTTTAA
 TTATTCCTAC GCTGCCGGA TCTCAGGAT TGCAGCCAA CGGCGGCCCG CAAAATAA CAATTGAGTA CAACTGTCTG AATAGTAGCT ATTGAAAT
 477 O
 3101 TCGGTAGTGT TATCAGATT AATTTGCTAA CGCAGTCAGG CACCGTGAT GAAATCTAAC AATGCGTCA TCGTCTATCT CGGCACCGTC ACCCTGGATG
 AGCCATCAA ATAGTGTCAA TTTAACGATT GCGTCAGTCC GTGGCACATA CTTAGATG TTACGGAGT AGCAGTGA GCGGTGGCAG TGGGACCTAC

^Start Tet Resistance Coding Sequence

FIG. 19D

3201 CTGTAGGCAT AGGCTTGGTT ATGCCGGTAC TGCCGGGGCCT CTTCGGGGAT ATCGTCCATT CCGACAGCAT CGCCAGTCAC TATGGCGTGC TGCTAGCGCT
GACATCCGTA TCCGAACCAA TACGGCCATG ACGGCCCCGA GAACGCCCTA TAGCAGGTAA GGCTGTGTA GCGGTGAGTG ATACCGCAGC ACGATCGCGA

3301

FIG. 20A

1 GAATTCACCT TCTCCATCT TTGGATAAGG AAATPACAGAC ATGAAAAATC TCATGTCTGA GTTGTATTTT AAGCTTGCCC AAAAAGAAGA AGAGTCGAAT
 CTTAAGTTGA AGAGGTATGA AACCTATTCC TTTAATGCTG TACTTTTAG AGTAACGACT CAACAATAAA TTCGAACGGG TTTTCTTCT TCTCAGCTTA
 101 GAACTGTGT GCGAGGTAGA AGCTTTGGAG ATATTCGTCA CTGCAATGCT TCGCAATPAT GCGCAAAATG ACCAACAGCG GTTGATTTGAT CAGGTAGAGG
 CTTGACACAC GCGTCCATCT TCGAAACCTC TAATAGCAGT GAGGTATACA AGCGTTATAC CGCGTTTAC TGGTTGTGCG CAACATACTA GTCCATCTCC
 201 GGGGCTGTA CGAGGTAAAG CCCGATGCCA GCATTCCTGA CGACGATACG GAGTGTGCTG CGGATPACGT AAAGAAGTTA TTGAAGCATC CTCGTACGTA
 CCCGCGACAT GCTCCATTTC GGGCTACGCT CGTAAGGACT GCTGCTATGC CTCGACGACG CGCTAATGCA TTTCTTCAAT AACTTCGTAG GAGCAGTCAT
 301 AAAAGTTAAT CTTTCAACA CTTGTCAATA AGTTGTACAG GCGGAGACTT ATAGTCGCTT TGTTTTATT TTTTAATGTA TTTGTAACTA GTACGCAAGT
 TTTTCAATTA GAAAAGTTGT CGACAGTATT TCAACAGTGC CGGCTCTGAA TATCAGCGAA ACATAAATAA AAAATTACAT AAACATTGAT CATGCCGTTCA
 401 TCACGTAAAA AGGGTATCTA GAATTATGAA GAAGAATATC GCATTTCTTC TTGCATCTAT GTTCGTTTTT TCTATTGCTA CAAAGCGGTA CGCTGATATC
 AGTGCAATTT TCCCATAGAT CTTAATACTT CTTCTTATAG CGTAAAGAAG AACGTAGATA CAAGCAAAAA AGATAACGAT GTTTGCGCAT GCGACTATAG
 1 M K K N I A F L L A S M F V F S I A T N A Y A D I
 ^STII signal TIR-1 anti-IgE light chain^
 501 CAGCTGACCC AGTCCCGAG CTCCTGTGCG GCGATAGGT CACCATACC TGCCGTGCCA GTCAGAGCGT CGATTACGAT GGTGATAGCT
 GTCGACTGGG TCAGGGGCTC GAGGACACAG CGCTATCCCA GTGGTAGTGG ACGGCACGGT CAGTCTCGCA GCTAATGCTA CCACTATCGA
 26 Q L T Q S P S L S A S V G D R V T I T C R A S Q S V D Y D G D S Y
 601 ACATGAAGTG GTATCAACAG AAACACAGGAA AAGCTCCGAA ACTACTGATT TACGCGCCT CGTACTGGA GTCTGGAGTC CTTTCTCGCT TCTCTGGATC
 TGTACTTGAC CATAGTTGTC TTTGGTCCCTT TTCGAGGCTT TGATGACTAA ATGCCCGGA GCATGGACCT CAGACCTCAG GGAAGAGCGA AGAGACCCTAG
 60 M N W Y Q Q K P G K A P K L L I Y A A S Y L E S G V P S R F S G S
 701 CGGTCTTGGG ACGGATTTCA CTCTGACCAT CAGCAGTCTG CAGCCGGAAG ACTTCGCAAC TTATTACTGT CAGCAAAAGTC ACGAGGATCC GTACACATTT
 GCCAAGACCC TGCCTAAAGT GAGACTGGTA GTCGTCAGAC GTCGGCTTC TGAAGGTTG AATAATGACA GTCTTTTCAG TGTCTCTAGG CATGTGTAAA
 93 G S G T D F T L T I S S L Q P E D F A T Y Y C Q Q S H E D P Y T F
 801 GGACAGGGTA CCAAGGTGGA GATCAACGA ACTGTGGCTG CACCATCTGT CTTCTATCTC CGCCATCTG ATGAGCAGTT GAAATCTGGA ACTGCCCTCTG
 CCTGTCCCAT GGTTCACCT CTAGTTTGCT TGACACCGAC GTGGTAGACA GAAGTAGAAG GCGGTAGAC TACTCGTCAA CTTTAGACCT TGACGGAGAC
 126 G Q G T K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V
 901 TTGTGTGCTT GCTGAATAAC TTCTATCCCA GAGAGGCCAA AGTACAGTGG AAGTGGATA ACGCCCTCCA ATCGGGTAAC TCCAGGAGA GTGTACAGA
 AACACACGGA CGACTTATG AAGATAGGCT CTCTCCGGTT TCATGTCACC TTCCACCTAT TGCCGAGGT TAGCCCATG AGGTCCTCT CACAGTCTCT
 160 V C L L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E

FIG. 20B

1001 GCAGGACAGC AAGACAGCA CCTACAGCCT CAGCAGCACC CTGACGCTGA GCAAAGCAGA CTACAGAGAA CACAAAGTCT ACGCTTGGGA AGTCACCCAT
 CGTCCCTGTCG TTCTCTGCTT GGAATGCGGA GTGCTGCTGG GACTGGGACT CGTTTCGTCT GATGCTCTTT GTGTTTCAGA TGGGAGACGT TCAGTGGGTA
 193 Q D S K D S T Y S L S S T L T L S K A D Y E K H K V Y A C E V T H
 1101 CAGGCGCTGA GCTCGCCCGT CACAAAGAGC TTCAACAGGG GAGAGTGTTA ATTAATCCT CTACGCCGGA CGATCGTGG CGAGCTCGGT ACCCGGGGAT
 GTCCCGGACT CGAGCGGCA GTGTTTCTCG AAGTTGTCCC CTCTACAAT TAAATTAGGA GATGGGCCCT GCGTAGCACC GCTCGAGCCA TGGGCCCCCTA
 226 Q G L S S P V T K S F N R G E C O
 1201 CTAGGCCTAA CGTCTGGTGG CCQCCGGGGC TTTTATTATG TGCCCGAGCG GCATCTCGAA TGAACCTGCT GCGCAGGTAG AAGCTTTGGA GATTATCGTC
 GATCCGGATT GCGAGCCAAAC GGCGGCCCGC AAAAAATAAC AACGGCTGCG CGTAGAGCTT ACTTGACACA CGCGTCCATC TTCGAAACCT CTAATAGCAG
 1301 ACTGCAATGC TTCGCAATAT GGGCGAAAT GACCAACAGC GGTGTGATTGA TCAGGTAGAG GGGGCGCTGT ACGAGGTAA GCGCGATGCC AGCATTCCTG
 TGACCTTACG AAGCGTTATA CCGCGTTTTA CTGTTGTGCG CCAACTAACT AGTCCATCTC CCGCGGACA TGTCTCATTT CCGGCTACGG TCGTAAGGAC
 1401 ACGACGATAC GGAGCTGCTG CCGGATTAGG TAAAGAAGTT ATTGAAGCAT CCTCGTCAGT AAAAGTTAA TCITTTCAAC AGCTGTCTATA AAGTTGTCAC
 TGCTGCTATG CCTCGACGAC GCGCTAATGC ATTTCTTCAA TAACTTCGTA GGAGCAGTCA TTTTTCATTT AGAAAAGTTG TCGACAGTAT TTCAACACAGTG
 1501 GGCCGAGACT TATAGTCGCT TTGTTTTTAT TTTTAAATGT ATTTGTAATCT AGTACGCAAG TTCACGTAA AAGGTATCT AGAATTATGA AGAAGATAT
 CCGGCTCTGA ATATCAGCGA AACAAAAATA AAAAATTACA TAAACATGTA TCATGCGTTC AAGTGCATTT TTCCCATAGA TCTTAATACT TCTTCTTATA
 M K K N I
 ^STII signal TIR-1
 1601 CGCATTTTCTT CTTCGATCTA TGTTTCGTTTT TTCTATTGCT ACAAAACGGT ACGCTGAGGT TCAGTGGTG GAGTGTGGG GTGGCCTGGT GCAGCCAGGG
 GCGTAAAGAA GAACGTAGAT ACAGCAAAA TGTTCGCGCA TCGCACTCCA AGTCCAGCAC CTCAGACCGC CACCGEACCA CGTCGGTCCC
 6 A F L L A S M F V F S I A T N A Y A E V Q L V E S G G L V Q P G
 ^anti-IgE heavy chain
 1701 GGCTCACTCC GTTTGTCCTG TGCAGTTTCT TGCACTCTCCG ATATAGCTGG AACTGGATCC GTCAGGCCCC GGTAAAGGC CTGGAATGGG
 CCGAGTGGG CAACAGGAC ACCTCAAAGA CCGATGAGGT AGTGGAGGCC TATATCGACC TTGACCTAGG CAGTCGGGG CCCATTCCCG GACCTTACCC
 39 G S L R L S C A V S G Y S I T S G Y S W N W I R Q A P G K G L E W V
 1801 TTGCAATCAT TACGTATGAC GGATCGACTA ACTATAACC TAGCGTCAAG GCGCGTATCA CTATAAGTCG CGACGACTCC AAAAACACAT TCTACCTGCA
 AACGTAGCTA ATGCATCTG CCTAGCTGAT TGATATTGGG ATCGCAGTTC CCGGCATAGT GATATTGAGC GCTGCTGAGG TTTTGTGTA AGATGGACGT
 73 A S I T Y D G S T N Y N P S V K G R I T I S R D D S K N T F Y L Q
 1901 GATGAACAGC CTGCTGCTG AGGACACTGC CGTCTATTAT TGTGCTCAG GCAGCCACTA TTTCGGTTCAC TGGCACTTCG CCGTGTGGGG TCAAGGAACC
 CTACTTGTG GACGACGAC TCCTGTGACG GCAGATAATA ACACAGCTC CCGTGGTGT AAGCCAGTG ACCGTGAAGC GGCACACCCC AGTTCCCTGG
 106 M N S L R A E D T A V Y Y C A R G S H Y F G H W H F A V W G Q G T
 2001 CTGGTCACCG TCTCTCTGCG CTCACCAAG GGCCCATCGG TCTTCCCTCT GGCACCTCC TCCAAGACA CCTCTGGGG CACAGCGGCC CTGGGCTGCC
 GACCACTGGC AGAGGACCG GAGTGGTTC CCGGCTAGCC AGAAGGGGGA CCGTGGGAGG AGGTTCTCGT GGAGACCCCC GTGTGCGCCGG GACCCGAGG
 139 L V T V S S A S T K G P S V F P L A P S S K S T S G G T A A L G C L

FIG. 20C

2101 TGGTCAAGGA CTACTTCCCG GAACCGGTGA CGGTGTCGTG GAACTCAGGC GCCCTGACCA GCGGGGTGCA CACCTTCCCG GCTGTCTTAC AGTCTTCAGG
 ACCAATTCCT GATGAAGGGG CTTGGCCACT GCACACGAC CTTGAGTCQG CCGGACTGGT CCGGCACAGT GTGGAGGGC CGACAGGATG TCAGGAGTCC
 173 V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S G
 2201 ACTCTACTCC CTCAGCAGCG TGGTACTGT GGCCTCTAGC AGCTTGGGCA CCCAGACCTA CATCTGCAAC GTGAATCACA AGCCACAGCAA CACCAAGGTG
 TGAATGAGG GAGTCGTGCG ACCACTGACA CCGGAGATCG TCGAACCCTG GGGCTGGAT GTAGAGTTG CACTTAGTGT TCGGGTCTGT GTGGTTCCAC
 206 L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V
 2301 GACAAGAAAG TTGAGCCCAA ATCTTGAC,AAAACCTACA CATGCCACC GTGCCAGCA CCTGAACCTC TGGGGGACC GTCACTCTTC CTCTTCCCCC
 CTGTCTTTC AACTCGGGT TACAACACTG TTTTGAATGT GTACGGGTGG CACGGGTCTG GCACTTGAGG ACCCCCTTGG CAGTCAGAAG GAGAAGGGG
 239 D K K V E P K S C D K T H T C P P C P A P E L L G G P S V F L F P P
 2401 CAACAACCCAA GGACCCCTC ATGATCTCC GGACCCCTGA GGTCAATGC GTGTGTGTG ACGTAGCCA CBAAGACCTT GAGGTCAAGT TCAACTGGTA
 GTTTTGGGTT CCTGTGGAG TACTAGAGG CCTGGGACT CCAGTGTAG CACCACCACC TGCACCTGGT GCTTCTGGGA CTTCCAGTTCA AGTTGACCAT
 273 K P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y
 2501 CGTGACGGC GTGAGGTGC ATAAATGCCAA GACAAAGCCG CGGGAGGAG AGTACAACAG CACCTACCTT GTGTACGGT TCCTCACCGT CCTGCACCAG
 GCACCTGCCG CACCTCCAG TATTACGTT CTGTTTCGGC GCCCTCTCTG TCATGTGTC GTGCATGGCA CACGATGCG AGGAGTGGCA GGACGTGGTC
 306 V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q
 2601 GACTGGCTGA ATGCAAGGA GTACAAGTGC AAGGTCTCCA ACAAAGCCT CCCAGCCCC ATCGAGAATA CCATCTCAA AGCCAAAGGG CAGCCCGGAG
 CTGACCGACT TACGGTCTT CATGTTACG TGTTCGGGA GGGTCGGGG TAGTCTTTT GTTAGAGTTT TCGTTTCCC GTCCGGGCTC
 339 D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E
 2701 AACACAGGT GTACACCCCTG CCCCCATCC GGAAGAGAT GACCAAGAC CAGTTCAGC TGACCTGCCCT GGTCAAAGGC TTCTATCCCA GGCACATCGC
 TTGTGTCCA CATGTGGAC GGGGGTAGGG CCCTTCTCTA CTGGTTCTTG GTCCAGTCGG ACTGCACGGA CCAATTTCCG AAGATAGGT CGCTGTAGCG
 373 P Q V Y T L P P S R E E M T K N Q V S L T C L V K G F Y P S D I A
 2801 CGTGGAGTGG GAGAGCAATG GGCAGCCGGA GAACAACCTAC AAGACACACG CTCCTCGTGT GCACTCCGAC GGTCTCTTCT TCTCTACAG CAAGTCAACC
 GCACCTCACC CTCTCGTTAC CCGTCGGCT CTGTGTGATG TTCTGTGTGG GAGGCACGA CCTGAGGCTG CCGAGGAGA AGGAGATGTC GTTCGAGTGG
 406 V E W E S N G Q P E N N Y K T T P P V L D S D G S F F L Y S K L T
 2901 GTGACAAGA GAGGTGGCA GAGGGGAAC GTCTTCTCAT GCTCCGTGAT GCATGAGGCT CTGCACAACC ACTACACGCA GAAGAGCCTC TCCCTGTCTC
 CACCTGTTCT GTCCACCGT CGTCCCTTG CAGAAGATA CGAGGACATA CGTACTCCGA GACGTGTGG TGAATGTGCT CTTCTCGAG AGGACAGAG
 439 V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P
 3001 CGGGTAAATA AGCATGCGAC GGCCCTAGAG TCCCTAACGC TCGGTGCGG CCGGCGGTT TTTATGTGA ACTCAATGTT GACAGCTTAT CATCGATAAG
 GCCCATTTAT TCGTACGCTG CCGGGAICTC AGGGATGCG AGCCACAGGC GGCCCGCAAA AATAACAAAT TGAATACAAA CTGTCCAATA GTAGCTATTG
 473 G K O
 3101 CTTTAATGCG GTAGTTTATC ACAGTTAAT TGCTAACGCA GTCAGGCACC GTGTATGAAA TCTAACAAATG CGCTCATCGT CATCTCGGC ACCGTCAACC
 GAAATACGC CATCAATAG TGTCAATTA ACAGTTGCGT CAGTCCGTGG CACATACITT AGATGTGTAC GCGAGTAGCA GTAGAGCCG TGGCAGTGGG

FIG. 20D

3201 TGGATGCTGT AGCATAGGC TTGGTTATGC CGGTACTGCC GGGCCTCTTG CGGATATCG TCCATTCCGA CAGCATCGCC AGTCACTATG GCGTGTGCT
ACCTACGACA TCCGTATCCG AACCAATACG GCCATGACGG CCCGGAGAAC GCCCTATAGC AGGTAAAGCT GTCGTACCG TCACTGATAC CGCACCAGCA

3301

FIG. 21A

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1  GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA GTTGTTATTT AAGCTTGCCC AAAAAAGAAG AGAGTCGAAT
   CTTAAGTTGA AGAGGTATGA AACCTATTCC TTATGTGCTG TACTTTTFTAG AGTAACGACT CAACAATAAA TTCGAACGGG TTTTCTCTCT TCTCAGCTTA

101  GAACGTGTGTG CGCAGGTAGA AGCTTTGGAG ATTATCTPCA CTGCAATGCT TCGCAATATG GCGCAAAAATG ACCAACAGCG GTTGATTGAT CAGGTAGAGG
   CTTGACACAC CCGTCCATCT TCGAAACCTC TAATAGCAGT GACGTTACGA AGCGTTATAC CCGGTTTTAC TGGTTGTCCG CAACCTAATA GTCCATCTCC

201  GGGCGCTGTA CGAGGTAAAG CCGGATGCCA GCATTCCTGA CGACGATPAC GAGCTGCTGC GCGATTACGT AAAGAAGTTA TTGAAGCATC CTCGTCAAGTA
   CCCGCGACAT GCTCCATTTC GGGCTACGGT CGTAAGGACT GCTGCTATGC CTCGACGACG CGCTAATGCA TTTCTTCAAT AACTTCGTAG GAGCAGTCAT

301  AAAAGTTAAT CTTTTCACA GCTGTCTATA AGTTGTACAG GCCGAGACTT ATAGTCGCTT TGTTTTATT TTTTAATGTA TTTGTAAGTA GTACGCCAAGT
   TTTTCAATTA GAAAGTTGCT CGACAGTATT TCAACAGTGC CGGCTCTGAA TATCAGCGAA AAAAAATAA AAATATACAT AAACATTGAT CATGCGTTCA

401  TCACGTAAAA AGGTATCTTA GAATTATGAA GAAGAATATC GCATTTCTTC TTGCATCTAT GTTCGTTTTT TCTATTGCTA CAAACGCCGA CGCTGATATC
   AGTGCATTTT TCCCATAGAT CTTAATACCT CTTCTTATAG CGTAAGAAG AACGTAGATA CAAGCAAAA AGATAACGAT GTTTGCGCAT GCGACTATAG
   1      M K K I A F L L A S M F V F S I A T N A Y A D I
           ^still signal TIR-1
           anti-IgE light chain^

501  CAGCTGACCC AGTCCCCGAG CTCCTGTCTC GCTCTGTGG GCGATAGGGT CACCATCACC TGCGGTGCCA GTCCAGAGCGT CGATTACGAT GGTGATAGCT
   GTCGACTGGG TCAGGGGGCTC GAGGGACAGG CCGAGACACC CGCTATCCCA GTGGTAGTGG ACGGCACGGT CAGTCTCCGA GCTAATGCTA CCACATACGA

26 Q L T Q S P S L S A S V G D R V T I T C R A S Q S V D Y D G D S Y

601  ACATGAAC TGATCAACAG AAACCAAGAA AAGCTCCGAA ACTACTGATT TACGCGGCCT CGTACCTGGA GTCTGGAGTC CCTTCTCGCT TCTCTGGATC
   TGTACTTGAC CATAGTTGTC TTTGGTCCCT TTTCGAGGCTT TGATGACTAA ATGCGCCGGA GCATGGACCT CAGACCTCAG GGAAGAGCGA AGAGACCTAG

60 M N W Y Q Q K P G K A P K L L I Y A A S Y L E S G V P S R F S G S

701  CGGTTCTGGG ACGGATTTCA CTCGACCAT CAGCAGTCTG CAGCCGGAAG ACTTCGCAAC TTATTACTGT CAGCAAAATC ACAGGATCC GTACACATTT
   GCCAAGACCC TGCTTAAAGT GAGACTGGTA GTCGTCAGAC GTCGGGCTTC TGAAGGTTG AATATGACA GTCGTTTCAG TGCCTCCTAGG CATGTGTAAA

93 G S G T D F T L T I S S L Q P E D F A T Y Y C Q Q S H E D P Y T F

801  GGACAGGGTA CCAAGGTGGA GATCAACGA ACTGTGGCTG CACCATCTGT CTTCATCTTC CCGCATCTG ATGAGCAGTT GAAATCTGGA ACTGCCCTCTG
   CCTGTCCCAT GGTTCACCT CTAGTTTGCT TGACACCGAC GTGGTAGACA GAAGTAGAAG GGGGTAGAC TACTCGTCAA CTTTAGACCT TGACGGAGAC

126 G Q G T K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V

901  TTGTGTGCCT GCTGAATAAC TTCTATCCCA GAGAGGCCAA AGTACAGTGG AAGGTGATA ACGCCTCCA ATCGGGTAAC TCCAGGAGA GTGTACAGA
   AACACACGGA CGACTTATG AAGATAGGTT CTCTCCGGTT TCATGTCACC TTCCACCTAT TGCGGAGGT TAGCCCATTG AGGTCCTCT CACAGTCTCT

160 V C L L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E

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FIG. 21B

1001 GCAGGACAGC AAGGACAGCA CCTACAGCCT CAGCAGCACC CTGAGGCTGA GCAAAGCAGA CTACGAGAAA CACAAAGTCT AGCCCTGCGA AGTCAACCAT
 CGTCCCTGTCG TTCCTGTGCT GGAATGCGA GTCGTGCTGG GACTGCGACT CGTTTCGCTT GATGCTCTTT GTGTTTCAGA TCGGAGCGCT TCAGTGGGTA
 193 Q D S K D S T Y S L S S T L T L S K A D Y E K H K V Y A C E V T H
 1101 CAGGGCCTGA GCTCGCCCGT CACAAAGAGC TTCAACAGGG GAGAGTGTTA ATTAAATCCT CTACGCCGGA CGCATCGTGG CGAGTCGGT ACCCGGGGAT
 GTCCCGGACT CGAGCGGGCA GTTCTTCTCG AAGTTGTGCC CTCTCACAAT TAATTAGGA GATCGGGCT GCCTAGCACC GCTCGAGCCA TGGGCCCTTA
 226 Q G L S S P V T K S F N R G E C O
 1201 CTAGGCCTAA CGCTCGGTTG CCGCCGGGCG TTTTATTATG TTGCCGAGCG GCACTCGAA TGAAGTGTGT GCGCAGGTAG AAGTTTGA GATTATCGTC
 GATCCGGATT GCGAGCCCAAC GCGCGCCCGC AAAAATAAAC AACGGCTGCG CGTAGAGCTT ACTTGACACA CGCGTCCATC TTCGAAACCT CTAATAGCAG
 1301 ACTGCAATGC TTCGCAATAT GCGGCAAAAT GACCAACAGC GGTGATTGA TCAGGTAGAG GGGGGGCTCT ACCAGGTAAA GCCCGATGCC AGCATTCCTG
 TGACGTTACG AAGGTTTATA CCGCGTTTGA CTGGTTGTCG CCAACTAACT AGTCCATCTC CCCCGCGACA TGCTCCATTT CGGGCTACGG TCGTAAGGAC
 1401 ACGACGATAC GGAGCTGCTG CCGGATTACG TAAAGAAGTT ATTGAAGCAT CCTGTCAGT AAAAAGTTAA TCTTTTCAC AGCTGTGATA AAGTTGTCAC
 TGCTGCTATG CCTCGACGAC GCGCTAATGC ATTCTTCAA TAACTTCGTA GGAGCAGTCA TTTTTCAAAT AGAAAAGTTG TCGACAGTAT TTCAACAGTG
 1501 GGCCGAGACT TATAGTCGCT TTGTTTATAT TTTTAAATGT ATTGTAACT AGTAGCAAG TTCACGTAAA AAGGTATCT AGAATTATGA AGAAGAATAT
 CCGGCTCTGA ATATCAGCGA AACAAAAATA AAAAATTACA TAAACATTGA TCATGCGTTC AAGTGCATTT TTCCCATAGA TCTTAATACT TCTTCTTATA
 M K K N I
 ^SII signal TIR-1
 1
 1601 CGCATTTCTT CTTCGATCTA TGTTCTGTTT TTCTATTGCT ACAAAACGGT ACGTCAAGT TCAGCTGCAA GAGTCTGGCC CGGGCCTGGT GAAACCATCT
 GCGTAAAGAA GAACGTAGAT ACAAGCAAAA AAGATAACGA TGTGTCGCA TCGAGTCCA AGTCAGCTT CTGAGACCGG GCCCGGACCA CTTTGGTAGA
 6 A F L L A S M F V F S I A T N A Y A Q V Q L Q E S G P G L V K P S
 ^anti-IgE Heavy Chain
 ^Heavy Chain FRI=SubgroupII consensus sequence
 1701 CAGACTCTCT CCTTGACTTG TACTGTTTCT GGCTACTCCA TCACCTCCGG ATATAGTGG AACTGGATCC GTACGGCCCC GGGTAAGGGC CTGGAATGGG
 GTCTGAGAGA GGAAGTGAAC ATGACAAAGA CCGATGAGGT AGTGGAGGCC TATATCGACC TTGACCTAGG CAGTCCGGGG CCCATPCCCG GACCTTACCC
 39 Q T L S L T C T V S G Y S I T S G Y S W N W I R Q A P G K G L E W V
 1801 TTGCAATCGAT TACGTATGAC GGATCGACTA ACTATAACCC TAGCGTCAAG GCGCGTATCA CTATAAGTCG CGACGACTCC AAAAACACAT TCTACCTGCA
 AACGTAGCTA ATGCATATG CCTAGCTGAT TGAATATTGG ATCGCAGTTC CCGGCATAGT GATATTCAGC GCTGCTGAGG TTTTGTGTA AGATGGACGT
 73 A S I T Y D G S T N Y N P S V K G R I T I S R D D S K N T F Y L Q
 1901 GATGAACAGC CTGCGTGTG AGGACACTGC CGTCTATTAT TGTCTCTGAG GCAGCCACTA TTTCCGTCAC TGGCACTTCG CCGTGTGGGG TCAAGGAACC
 CTACTTCTCG GACGCACGAC TCCTGTGACG GCAGATAATA ACAGGACTC CGTCCGTGAT AAAGCCAGTG ACCGTGAAGC GGCACACCCC AGTTCCTTGG
 106 M N S L R A E D T A V Y Y C A R G S H Y F G H W H F A V W G Q G T
 2001 CTGGTCAACG TCTCTTCGGC CTCCACCAAG GGCCCATCGG TCTTCCCTT GGCACCTCTCC TCCAAGAGCA CCTCTGGGGG CACAGCGGC CTGGGCTGCC
 GACCAATGGC AGAGGAGCG GAGTGGTTC CCGGTAGCC CCGTGGAGGA CCGTGGAGG AGGTTCTCGT GGAGACCCCC GTGTGCGCGG GACCCGACGG
 139 L V T V S S A S T K G P S V F P L A P S S K S T S G G T A A L G C L

FIG. 21C

2101 TGGTCAAGGA CTACTTCCCC GAACGGGTGA CGGTGTCTGT GAACTCAGGC GCCCTGACCA GCGGCCTGCA CACCTTCCCG GCTCTCTTAC AGTCTCTCAGG
 ACCAGTCTCT GATGAAGGGG CTTGGCCACT GCCACAGCAC CTTGAGTCCG CCGGACTGGT CCGCGACCGT GTGGAAGGCG CGACAGGATG TCAGGAGTCC
 173 V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S G
 2201 ACTCTACTCC CTCAGCAGCG TGGTACTGT GCCCTCTAGC AGTTTGGCA CCCAGACCTA CATCTGCAAC GTGAATCACA AGCCAGCAA CACCAAGGTG
 TGAGATGAGG GAGTCGTCCG ACCACTGACA CCGGAGATCG TCGAACCCGT GGTCTCTGAT GTAGACGTTG CACTTAGTGT TCGGTCTGTT GTGGTTCAC
 206 L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V
 2301 GACAAGAAAG TTAGGCCAA ATCTGTGTGAC' AAAACTCACA CATGCCACC GTGCCAGCA CCTGAATCC TGGGGGACC GTCACTCTTC CTCTTCCCC
 CTGTTCTTTC AACTCGGGT TAGAACACTG TTTTGAGTGT GTACGGGTGG CACGGGTCTG GACTTGAAG ACCCCCTGG CAGTCAGAAG GAGAAGGGG
 239 D K K V E P K S C D K T H T C P P C P A P E L L G G P S V F L F P P
 2401 CAAAACCCAA GGACACCCCTC ATGATCTCCC GGACCCCTGA GGTCAATGC GTGGTGTGG AGTGAAGCCA CGAAGACCCCT GAGTCAAGT TCAACTGGTA
 GTTTTGGGTT CCTGTGGAG TACTAGAGG CCTGGGGACT CCAAGTGTACG CACCACCACC TGCACCTGGT GCTTCTGGA CTCCAGTTCA AGTTGACCAT
 273 K P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y
 2501 CGTGGACGGC GTGGAGTGC ATAATGCCAA GACAAAGCCG CCGGAGGAGC AGTACAACAG CACGTACCGT GTGTCAGCG TCCTCACCGT CTGCAACCAG
 GCACCTGCCG CACCTCCACG TAITACGGTT CTGTTTCGGC GCCCTCTCTG TCATGTTGTC GTGCATGGCA CACCACTCGC AGGAGTGGCA GGAGTGTGTC
 306 V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q
 2601 GACTGTGTGA ATGGCAAGGA GTACAAGTGC AAGTCTTCCA ACAAGCCCTC CCAGCCCC ATCGAGAAA CCATCTCCA AGCCAAAGG CAGCCCCGAG
 CTGACCGACT TACCGTTCTC CATGTTACG TTTCCAGAGT TGTTCGGGA GGGTCGGGG TAGCTCTTTT GTAGAGGTT TCGGTTTCCC GTCGGGGCTC
 339 D W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E
 2701 AACCACAGGT GTACACCCCTG CCCCATCCC GGAAGAGAT GACCAAGAAC CAGTCAAGC TGACCTGCCT GGTCAAAGGC TTCTATCCCA GCGACATCGC
 TTGGTGTCCA CATGTGGAC GGGGTAGGG CCTTCTCTA CTGTTCTTG GTCCAGTCGG ACTGGAAGCA CCAAGTTCCG AAGATAGGTT CGCTGTAGCG
 373 P Q V Y T L P P S R E E M T K N Q V S L T C L V K G F Y P S D I A
 2801 CGTGGAGTGG GAGAGCAATG GGCAGCCGGA GAACAACCTAC AAGACCAGC CTCCCCTGCT GGAATCCGAC GGTCTCTTCT TCCTCTACAG CAGCTCACC
 GCACCTCACC CTCCTGTTAC CCGTCGGCCT CTTGTTGATG TTCTGGTGG GAGGACAGA CCTGAGGCTG CCGAGGAGA AGGAGATGTC GTTCGAGTGG
 406 V E W E S N G Q P E N N Y K T T P P V L D S D G S F F L Y S K L T
 2901 GTGGAAGA GCAGTGGCA GCAGGGGAC GTCTTCTCAT GTCCTGTAT GCATGAGGCT CTGCACAACC ACTACAGCA GAGAGCCCTC TCCTGTCTC
 CACCTGTCT CGTCCACCGT CGTCCCCTTG CAGAAGATA CGAGGCACTA CGTACTCCGA GACGTGTGG TGAATGCGT CTTCTCGGAG AGGACAGAG
 439 V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P
 3001 CGGGTAAATA AGCATGCGAC GGCCCTAGAG TCCTAAGC TCGTTGCCG CCGGGGTTT TTTATGTTA ACTCATGTT GACAGCTTAT CATCGATAAG
 GCCCATTTAT TCGTACGCTG CCGGATCTC AGGGATTGCG AGCCAACGGC GGCCCCAAA AATAACAAT TGAGTACAAA CTGTGGAATA GTAGCTATTC
 473 G K O
 3101 CTTTAAATCG GTAGTTTATC ACAGTTAAAT TGCTAACGCA GTCAAGCACC GTGTATGAA TCTAACAAT CGCTCATCGT CATCTCCGC ACCGTACCC
 GAATATACGC CATCAAAATG TGTCAATTTA ACGATTGCGT CAGTCCCTGG CACATACTTT AGATTGTTAC CGAGTAGCA GTAGGAGCGG TGGCAGTGGG

FIG. 21D

3201 TGGATGCTGT AGGCATAGGC TTGGTTATGC CGGTACTGCC GGGCCTCTTG CGGATATCG TCCATTCCGA CAGCATGCC AGTCACTATG GCGTCTGCT
ACCTACGACA TCGTATCCG AACCAATACG GCCATGACGG CCCGAGAAC GCCCTATAGC AGGTAAGGCT CTCGTAGCG TCAGTGATAC CGCAGACGA

3301

FIG. 22A

1 GAATTCACCT TCTCCATCT TTGGATAAGG AATATCAGAC ATGAAAAATC TCATTGCTGA GTTGTATT TT AGCTTGCC AAAAAGA AGAGTCGAAT
 CTTAAGTTGA AGAGGTATGA AACCTATCC TTATGTCGT TACTTTTAG AGTAAGACT CAACAATAA TTGGAACGGG TTTTCTCTCT TCTCAGCTTA
 101 GAACGTGTG CGCAGGTAGA AGCTTTGAG ATTATCGTCA CTGCAATGCT TCGCAATATG GCGCAAAATG ACCAACACAGG GTTGATTGAT CAGGTAGAGG
 CTTGACACAC CGGTCCATCT TCGAAACCTC TAATAGCAGT GACGTTACGA AGCGTTATAC CGCGTTTAC TGCTTGTCG CAACTAACTA GTCCATCTCC
 201 GGGCGCTGTA CGAGGTAAAG CCCATGCTGA GCATTCTCTGA CGACGATACG GAGCTGCTGC GCGATTACGT AAACAAGTTA TTGAAGCATC CTCGTGAGTA
 CCCGCGACAT GCTCCATTTT GGGCTACGCT CGTAAGGACT GCTGCTATGC CTCGACGACG CGCTAATGCA TTTCTTCAAT AACTTCGTAG GAGCAGTCAT
 301 AAAAGTTAAT CTTTTCACCA GCTGTCATAA AGTTGTCAG GCGGAGACTT ATAGTCGCTT TGTGTTTATT TTTTAATGTA TTGTAACTA GTACGCAAGT
 TTTTCAATTA GAAAGTTTCT CGACAGTATT TCAACAGTGC CGGCTCTGAA TATCAGCGAA ACAAAATAA AAAATTACAT AAACATTGAT CATGCGTTCA
 401 TCACGTAATA AGGTATCTA GAATTAATGAA GAAGAATATC GCATTTCTTC TTGCATCTAT GTTCGTTTTT TCTATGCTA CAAACGGGTA CGCTGATATC
 AGTGCATTTT TCCCATAGAT CTTAATACTT CTTCTTATAG CGTAAGAAG AACGTAGATA CAAGCAAAA AGATAACGAT GTTTGCGCAT GCGACTATAG
 1 M K K N I A F L L A S M F V F S I A T N A Y A D I
 ^STII Signal TIR -1 anti-VEGF Light Chain (1st generation)^

501 CAGATGACC AGTCCCGGAG CTCCTCTGCC GCGATAGGT CACCATCAC TGCAGCGCAA GTCAGCATAT TAGCAACTAT TTAACCTGGT
 GTCTACTGG TCAGGGGCTC GAGGACACG CGGAGACACC CGCTATCCCA GTGGTAGTGG ACGTCGCGTT CAGTCCTATA ATCGTTGATA AATTTGACCA
 26 Q M T Q S P S L S A S V G D R V T I T C S A S Q D I S N Y L N W Y
 60 Q Q K P G K A P K V L I Y F T S S L H S G V P S R F S G S G S G T
 TAGTTGCTTT TGTCTCTTTT CGAGGCTTTC ATGACTAAAT GAAGTGGAG AGAGAGTGA GACCTCAGG ARGAGCGAAG AGACCTAGGC CAAGACCTG
 601 ATCAACAGAA ACCAGGAAA GCTCCGAAAG TACTGATTTA CTTCACTCC TCTCTCCACT CTGAGATCCC TTCTCGCTTC TCTGATCCG GTTCTGGAC
 CCTAAGTGA GACTGTTACT CFTCAGACT CGGTCTCTG AAGCGTTGAA TAATGACAGT TGTCTATCG TGGCACGGCA CTGCAAAAC TGTCCCATGG
 93 D F T L T I S S L Q P E D F A T Y Y C Q Q Y S T V P W T F G Q G T
 701 GGATTTCACT CTGACCATCA GCGTCTGCA GCCAGAGAC TTCGCAACTT ATTACTGTC AACTATAGC ACGTGCCTT GGACGTTTGG ACAGGTACC
 AAGTGGAGA TCAACAGAAC TTGCTGCTGA CCATCTGTCT TCATCTTCCC GCCATCTGAT GAGCAGTTGA AATCTGGAAC TGCTTCTGTT GTGTGCTGTC
 801 TCCACCTCT AGTTGCTTG ACACCGAGCT GGTAGACAGA AGTAGAGGG CGGTAGACTA CTCGTCACT TTAGACCTTG ACGAAGCAA CACACGAGC
 126 K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L L
 901 TGAATACTT CTATCCCA GAGGCCAAG TACAGTGA GGTGATAAC GCCCTCCAAT CGGCTAATC CCAGGAGAGT GTCACAGAGC AGGACAGCAA
 ACTTATGAA GATAGGGTCT CTCGGGTTT ATGTCACTT CCACCTATTG CGGAGGTTA GCCATTGAG GTTCTCTCA CAGTGTCTCG TCCTGTCTGT
 160 N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K

FIG. 22B

1001 GGACAGCACC TACAGCCTCA GCAGCACCCCT GACGCTGAGC AAAGCAGACT ACAGAAACA CAAAGTCTAC GCTGCGAAG TCACCCATCA GGGCCTGAGC
 CCTGCTGCTG ATGTCGAGT CGTCGTGGA CTGCGACTCG TTTCCTCTGA TGCTCTTTGT GTTTCAGATG CCGACGCTTC AGTGGGTAGT CCGGACTCG
 193 D S T Y S L S S T L T L S K A D Y E K H K V Y A C E V T H Q G L S
 1101 TCGCCCGTCA CAAAGAGCTT CAACAGGGA GAGTGTAAAT TAAATCCTCT ACGCCGAGC CATCGTGCGG AGCTCGGTAC CCGGGGATCT AGGCCTAAGC
 AGCGGCACT GTTCTCTGAA GTTCTCCCT CTCACAATTA ATTTAGGAGA TCGGCGCTGC GTAGCACCGC TCGAGCCATG GGGCCCTAGA TCCGGATTGC
 226 S P V T K S F N R G E C O
 1201 CTGGTTGCC GCGGGGCTT TTTTATGTT GCCGACGCG ATCTCGAATG AACTGTGTC GCAGGTAGAA GCTTTGGAGA TTATCGTCAC TGCAATGCTT
 GAGCCACCG CGGCCCGCAA AAAATAACAA CCGCTGCGC TAGAGCTTAC TTGACACACG CGTCCATCTT CGAAACCTCT AATAGCAGTG ACGTTACGAA
 1301 CGCAATATGG CGCAAAATGA CCAACAGCGG TTGATTGATC AGGTAGAGGG GCGCTGTGAC GAGGTAAAGC CCGATGCCAG CATTCCTGAC GACGATACGG
 GCGTTATACC GCGTTTACT GGTGTGCGC AACTAATAG TCCATCTCC CCGGACATG CTCCATTTCG GGTACGGTC GTAAGGACTG CTGCTATGCC
 1401 AGCTGCTGCG CGATTACGTA ABAAGTTAT TGAAGCATCC TCGTCAGTAA AAGTTAATC TTTTCAACAG CTCTCATAAA GTTGTACAGG CCGAGACTTA
 TCGAGGACCG GCTAAATGCAT TTCTTCAATA ACTTCGTAGG AGCAGTCATT TTTCATTTAG AAAAGTTGTC GACAGTATTT CAACAGTGCC GGTCTGTAAT
 1501 TAGTCGCTTT GTTTTATTT TTAAATGAT TTGTAATAG TACGCAAGTT CACGTAAAAA GGGTATCTAG AATTATGAG AAGAATATCG CATTTCTTCT
 ATCAGCGAAA CAAAAATAAA AAATTACATA AACATTGATC ATGCGTTCAA GTGCATTTT CCATAGATC TTAATATCTT TTCTTATAGC GTAAAGAAGA
 1 M K K N I A F L L
 ^StII Signal TIR-1
 1
 1601 TGCATCTATG TTCGTTTTTT CTATTGCTAC AAACGCTAC GCTAGGTTT AGCTGGTGA GTCTGCGGT GGCCTGTGTC AGCCAGGGG CTCACTCCGT
 ACGTAGATAC AAGCAAGACC GATATGGAAG TTGCGCATG CGACTCCAAG TCGACCACT CAGACCGCCA CCGGACCAAG TCGTCCCCC GAGTGAGGCA
 10 A S M F V F S I A T N A Y A E V Q L V E S G G L V Q P G G S L R
 ^anti-VBGF (1st generation) heavy chain
 1701 TTGCTCTGTG CAGCTTCTGG CTATACCTTC ACCAATATG GTATGAATG GTTCGTCTAG GCCCGGGTA AGGECCTGGA ATGGTTGGA TGGATTAAACA
 AACAGGACAC GTCGAAGACC GATATGGAAG TTGCTGATAC CATACTTGAC CCAGGAGTC CCGGCCCCAT TCCCGGACCT TACCCAACT ACCTAATGT
 43 L S C A A S G Y T F T N Y G M N W V R Q A P G K G L E W V G W I N T
 1801 CCTATACCGG TGAACCGACC TATGCTGCGG ATTTCAAACG TCGTTTCACT TTCAGTTAG ACACCTCCA GTCCACAGCA TACCTGCAGA TGAACAGCCT
 GGATATGCC ACTTGGCTGG ATACGACGCC TAAAGTTGCG AGCAAGTGA AAGTCAATC TGTGAGGTT CAGCTGTCT ATGACAGTCT ACTTGTGGA
 77 Y T G E P T Y A A D F K R R F T F S L D T S K S T A Y L Q M N S L
 1901 CCGTGTCTGAG GACACTGCCG TCTATTACTG TGCAAAAGTAC CCCCACATAT ATGGAGCAG CCATGTGTAT TTCGACGTTT GGGGTCAAGG AACCTTGGTC
 CGCACGACTC CTGTGACGCG AGATATGAC AGTTTTCATG GGGGTGATAA TACCTCTGTC GGTGACATA AAGTGCAGA CCCAGTTCC TTGGGACGAG
 110 R A E D T A V Y Y C A K Y P H Y Y G S S H W Y F D V W G Q G T L V
 2001 ACCGTCTCCT CGGCTCCAC CAAGGGCCCA TCGGTCTTCC CCCTGGACC CTCCTCAAG AGCACTCTG GGGCACAGC GGCCTGGG TGCTGTGTC
 TGGCAGAGGA GCCGAGGTG GTTCCCGGT AGCCAGAAG GGGACCTGG GAGGAGGTTC TCGTGAGAC CCCGTGTG CCGGACCG ACGGACAGT
 143 T V S S A S T K G P S V F P L A P S S K S T S G T A A L G C L V K

FIG. 22C

2101 AGGACTACTT CCCCAGACCG GTGACGCTGT CGTGAACATC AGGCGCCCTG ACCAGCGGGG TGCACACCTT CCGGGCTGTC CTACAGTCCT CAGGACTCTA
 177 TCTGATGAA GGGGCTTGGC CACTGCCACA GCACCTTGAG TCCCGGGGAC TGGTGGCCGC ACCTGTGAA GGGCCGACAG GATGTCAGGA GTCTTGAGAT
 D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S G L Y
 2201 CTCCCTCAGC AGCGTGCTGA CTGTGCCCTC TAGAGCTTG GGCACCCAGA CCTACATCTG CAACGTGAAT CACAAGCCCA GCAACACCAA GGTGACACAAG
 GAGGAGTGG TCGCACCACT GACACGGGAG ATCGTCGAAC CCGTGGGTCT GGATGTAGAC GTTGCACTTA GTGTTGGGT CGTTGGGT CCACCTGTTT
 210 S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K
 2301 AAAGTTGAGC CCAATCTTG TGAACAAACT CACACATGCC CACCGTGCC AGCACCTGAA CTCTCTGGG GACCGTCAGT CTTCCTCTTC CCCCACAAAC
 TTTCACATCG GGTTAGAAC ACTGTTTGA GTGTGTACGG GTGGCACGGG TCGTGGACTT GAGGACCCCT CTGGCAGTCA GAAGAGAGAAG GGGGTTTIG
 243 K V E P K S C D K T H T C P P C P A P E L L G G P S V F L F P P K P
 2401 CCAAGGACAC CCTCATGATC TCCCGACCC CTGAGGTCAC ATGCGTGGTG GTGGACGTGA GCCACGAAGA CCTGAGGTC AAGTTCAACT GGTACGTGGA
 GGTTCCTG TGAGTACTAG AGGGCCTGGG GACTCCAGTG TACGCACACAC CACCTGCACT CGGTGCTTCT GGGACTCCAG TTCAAGTTGA CCATGCACCT
 277 K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y V D
 2501 CGCGTGGAG GTGCATAATG CCAAGACAAA GCCGGGGAG GAGCAGTACA ACAGCACGTA CCGTGTGTC AGGCTCTCTCA CCGTCTGCA CCAGSACTGG
 GCCGACCTC CAGGTATAC GGTTCGTTT GGGGCCCTC CTCGTCATG TGTGCTGAT GGCACACACAG TCGCAGGAGT GGCAGGACGT GGTCTGTGAC
 310 G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W
 2601 CTGAATGGA AGGAGTACAA GTGCAGGTC TCCAACAAAG CCGTCCCGC CCGCATCGAG AAAACCATCT CCAAGGCCAA AGGGCAGCCC CGAGAACCAC
 GACTTACCGT TCCTCATGTT CACGTTCCAG AGGTGTTTC GGGAGGTCG GGGTAGCTC TTGTGTAGA GGTTCGGT TCCCGTCGG GCTCTTGGT
 343 L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q
 2701 AGGTGTACAC CCGTCCCCA TCCCGGAAG AGATGACCAA GAACAGGTC AGCTGACCT GCGTGTCAA AGGCTTCTAT CCCAGCACA TCGCGTGA
 TCCACATNG GGACGGGGT AGGGCCCTC TCTACTGGT CTGTGTCAG TCGGACTGGA CGGACACAGT TCCGAAGATA GGTTCGCTGT AGCGCACCT
 377 V Y T L P P S R E E M T K N Q V S L T C L V K G F Y P S D I A V E
 2801 GTGGAGAGC AATGGGAGC CGGAGACAA CTACAGACC ACGCTCCCG TGTGAGCTC CGACGGCTCC TTCTTCTCT ACAGCAAGCT CACCGTGGAC
 CACCTCTCG TTACCCCTCG GCTCTTGT GATGTCTGG TCGGAGGGC ACACCTGAG GGTGCCGAGG AAGAAGAGA TGTCTGTCGA GTGGACCTG
 410 W E S N G Q P E N N Y K T T P P V L D S D G S F F L Y S K L T V D
 2901 AAGAGCAGGT GGCAGCAGG GAACGTCTTC TCATGCTCG TGATGCTGA GGCTGTGCAC AACCACATCA CGCAGAGAG CCTCTCCCTG TCTCGGGTA
 TTCTGCTCA CCGTCGTCCT CTGTGCAAG AGTACGAGC ACTAGTACT CCGAGACGTG TTGGTGATG GGTCTCTC GGAGAGGAC AGAGGCCCAT
 443 K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G K
 3001 AATAAGCAT CGACGGCCCT AGAGTCCCTA ACGTCCGTT GCCGCCGGG GTTTTTTATT GTTAATCTCAT GTTTGACAGC TTATCATCGA TAAGCTTTAA
 TTATTCGTAC GCTGCCGGA TCTCAGGAT TCGAGGCCA CGGGGCCCG CAAAAATAA CAATTGAGTA CAAACTGTCT AATAGTAGCT ATTGAAAT
 477 O
 3101 TCGGTAGTT TATCAGATT AAATGCTAA CGCATCAGG CACCGTGTAT GAAATCTAAC AATGCGTCA TCGTCACTCT CGGCACCGTC ACCCTGGATG
 ACGCCATCA ATAGTGTCAA TTAAAGATT CGGTAGTCC GTGCACATA CTTTAGATTG TTACGCCAGT AGCAGTAGGA GCCGTGGCAG TGGGACCTAC
 3201 CTGTAGCAT AGGCTTGGT ATGCGGTAC TCGCGGCCCT CTTGCGGAT ATGCTCCATT CCGACAGCAT CGCCAGTCAC TATGGGTGC TGCTAGCGCT
 GACATCCGTA TCCGAACCAA TACGGCCATG ACGGCCCGA GAACGCCCTA TAGCAGGTAA GGTGTCTGA GCGGTCTAGT ATACCGCAG ACGATCGCA

FIG. 23A

>Anti-VEGF (VITERX version) IgG1 Expression Cassette with heavy chain FR1,2,3,4=consensus subgroup1

earI/ksp632I
 bsrDI
 hindIII
 earI/ksp632I

1 GAATTCGAATTC TCTCCATACCT TTGGATAGAG AAATACAGAC ATGAAAATC TCATTGCTGA GTTGTATTATTT AAGCTTGCCC AAAAAGAAGA AGAGTCGAAT
 CTTAAGTTGA AGAGGTAGGA AAGCTATATCC TTATGTCTG TACTTTTAG AGTAACGACT CAACAATATAA TTGGAACGGG TTTTCTTCT TCTCAGCTTA

bamHI
 avdII/lopi hindIII
 bsrDI
 bclI

101 GAAGTGTGAG CGCAGCGAGA AGCTTTGAG AGTTATGTTCA CTGCAATGCT TCGCAATATG CGGCAAAATG ACCAACAGCG GTTGATTGAT CAGGTAGAGG
 CTTGACACAC CGCTCCATCT TCGAAAACCTC TAATAGCAGT GAGGTTACGA AGCGTTATAC CGCGTTTATC TGGTTGTCGC CAACTAACTA GTCCATCTCC

bamHI
 ahdII/eam1105I
 eagI/xmaIII/ec1XI
 speI

201 GGGCGCTGA GAGGTAGAG CGCGATATCCA GCATTCTCTGA CGACGATACG GAGCTGCTGC GCGATTACGT AAAGAAGTTA TTGAAGCATC CTCGTCAGTA
 CCGCGACAT CGTCCATTC CGCTACCGT CGTAAGGACT GCTGCTATGC CTCGACGACG CGCTAATGCA TTCTTCAAT AACTTCGTAG GAGCAGTCAT

pvuII
 ahdII/eam1105I
 eagI/xmaIII/ec1XI
 speI

301 AAAAGTTAAT GTTTTCACA GCTGTACAAA AGTTGTACG GCCGAGACTT ATAGTCGCTT TGTTTTTATT TTTTAATGTA TTGTAACTA GTACGCAAGT
 TTTTCATTA GAAAAGTTGT GCACAGTATT TCAACAGTGC CGGCTCTGAA TATCAGCGAA ACAAAAATAA AAAATTACAT AAACATTGAT CATGGGTTCA

bsiWI/splI
 mluI
 ecorV

401 TCACGTAATA ACCGATATCA GAATTATGAA GAAGAATATC GCATTCTTTC TTGCATCTAT GTTCGTTTTT TCTATTGCTA CAAACGGTA CGTCATATC
 AGTCATATT TCCCATAGAT CTAAATACCT CTTCTTATAG CGTAAAGAAG AACGTAGATA CAAGCAAAAA ACATAACGAT GTTTCGCGAT GCGACTATAG

1 M K K N I A F L L A S M F V F S I A T N A Y A D I
 ^STII Signal TIR -1 ^Light chain

501 CAGTTCACCC AGTCCGCGAG CAGCTGATCC GCTCTGTGAG GCGATAGGT CACCATCAC TCGACGCCAA GTCAGGATAT TAGCAACTAT TTAAGCTGGT
 GTCACACTGG TCGGCGCTC GAGGACAGG CGGAGACACC CGCTATCCCA GTGGTAGTGG AGCTCCCGTT CAGTCCCTATA ATCGTTGATA AATTGACCA

26 Q L T Q S P S S L S A S V G D R V T I T C S A S Q D I S N Y L N W Y

bstEII
 bspMI
 psti
 ahaIII/draI

601 ATCAACAGAA ACCAGCAAAA CGTCCGAAAG TACTGATTA CTTACCTCC TCTCTCCACT CTGGAGTCCC TTCTCGCTTC TCTGGATCCG GTTCTGGGAC
 TAGTTGATCT TGTGCTCTT CAGGCTTTC ATGACTAAT GAAGTGGAGG AGAGAGGTGA GACCTCAGG AGAGCGAAG AGACCTAGGC CAAGACCTG

bamHI
 bseRI
 bpmI/gsuI

60 Q Q K P Q K A P K V L I Y F T S S L H S G V P S R F S G S G S G T

kpnI
 asp718
 acc65I
 701 GGATTTCACT CTGACCAATCA GCAGTCCTCA CCCAGGAGC TTCCCAACTT ATTACTGTCA ACAGTATACC ACCGTGCCGT GGACGTTTGG ACAGGGTACC
 CCTAAAGTGA GACTGTAGT CCGTCACAGT CCGTCACAGT CCGTCACAGT CCGTCACAGT CCGTCACAGT CCGTCACAGT CCGTCACAGT CCGTCACAGT
 93 D F T L T I S E L Q P E D F A T Y Y C Q Q Y S T V P W T F G Q G T
 xmnI
 bpuAI
 bbsI
 801 AAGGTGAGCA TCAAAACCAAC TGTGCTCTCA CCATCTGTCT TCATCTTCCC GCCATCTGAT GAGCAGTCA AATCTGGAAC TGCTTCTGTT GTGTGCTGTC
 TTCCACCTCT AGTTTCTCTG ACACCCAGCT CCGTCACAGT CCGTCACAGT CCGTCACAGT CCGTCACAGT CCGTCACAGT CCGTCACAGT CCGTCACAGT
 126 K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L L
 xmnI
 asp700
 901 TGAATACTT CTATCCCAAC GAGCCCAAG TACAGTGAAG GGTGGATAAC GCCCTCCAAT CCGGTAACTC CCAGGAGAGT GTCACAGAGC AGGACAGCAA
 ACTTATTGAA GATAGGCTT CTGCTGCTT CCGCTGCTT CCACCTATTG CCGGAGGTTA GCCCATTTGAG GGTCTCTCTCA CAGTGTCTCG TCCTGTGCTT
 160 N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K
 sstI
 sacI
 celII/espI
 alwNI
 ecl136II
 blpI/bpu102I
 1001 GGACAGCACC TACAGCCTCA GAGCAGCCTT GAGCCTGAGC AAGCAGACT ACAGAGAAACA CAAAGTCTAC CCCTGCGAAG TCACCCATCA GGCCTGAGC
 CCTGTCTGAG ATGTCTGAGT CCGTCACAGT CCGTCACAGT CCGTCACAGT CCGTCACAGT CCGTCACAGT CCGTCACAGT CCGTCACAGT CCGTCACAGT
 193 D S T Y S L S E T L T L S K A D Y E K H K V Y A C E V T H Q G L S
 xmaI/pspAI
 kpnI
 sstI
 asp718
 pacI
 acc65I
 ecl136II
 smaI
 1101 TCGCCCTCA CAAACAGCTT CAAACAGCTT CAAACAGCTT CAAACAGCTT CAAACAGCTT CAAACAGCTT CAAACAGCTT CAAACAGCTT CAAACAGCTT
 AGCGGCGAGT GTTCTCTGAA GTTCTCTGAA GTTCTCTGAA GTTCTCTGAA GTTCTCTGAA GTTCTCTGAA GTTCTCTGAA GTTCTCTGAA GTTCTCTGAA
 226 S P V T K S F N R G E C O
 start lambda to terminator
 bspMI
 avII/fspI
 hindIII
 1201 CTGCGTTGCC GCGCGCGCTT TTTTATGTTT GCGGACGCGC ATCTCGAATG AACTGTGTGC GCAGGTAGAA GCTTTGGAGA TTATCGTCAC TGCATGCTT
 GAGCCACAGC GCGCCCGCAA AAAATAGAA CCGCTGCGG TAGAGCTTAC TTGACACACG CCGTCATCT CCAACCTCT AATAGCAGTG AGTTACGAA
 ^end lambda to terminator

FIG. 23C

FIG. 23D

3301

aatII (GACGTC) :	1983
acc65I (GGTACC) :	795 1176
ageI (ACCGGT) :	1806 2126
abaIII (TTTAAA) :	590
abdI (GACNNNNCTC) :	246 1495 2280
alw26I (CAGNNCTG) :	1089 1770 2259
alv44I (GTGAC) :	1930 2169

FIG. 23E